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Dietary Fermentable Fibre Affects Immune Function in BB rats, an Animal Model of Type-1 Diabetes

by

Rose Marie Stillie

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

in

Nutrition and Metabolism

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Dr. Karen Madsen

March 14th 2003

Abstract

Feeding a mixed, cereal-based (chow) diet to diabetes-prone BB (BBdp) rats results in a high incidence of diabetes compared to feeding a semi-purified, casein-based (control) diet. Neither the diet ingredient, nor the mechanism to explain this observation has been determined. The objective of this thesis research was to compare the immune response of BBdp and non-diabetes-prone BB (BBn) rats when fed a control diet, or a diet containing fermentable fiber (FF diet) from weaning (21 days of age) until 35 days-of-age. FF feeding significantly increased gut length (P=0.0031), CD8+ cells (P=0.003) in the Peyer's Patches and mitogen stimulated proliferation (P=0.038) in normal rats, but not BBdp rats. Feeding FF significantly increased the proportion of IgA+ cells in the lamina propria (P=0.0036) and B-cells in the Peyer's Patches (P=0.01) in BBdp rats but not BBn rats. Diet did not affect the expression of CD80 in the gut. This study has demonstrated that BBdp rats do not respond the same as BBn rats when challenged with a FF diet at weaning.

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List of Abbreviations

APC	Antigen-Presenting Cell
BB	BioBreeding
BBdp	BioBreeding Diabetes Prone
BBn	BioBreeding Normal
BSA	Bovine Serum Albumin
CD	Cluster Designation
DC	Dendritic Cell
ConA	Concanavalin A
FF	Fermentable Fibre
GAD	Glutamic Acid Decarboxylase
GALT	Gut-Associated Lymphoid Tissue
GI	GastroIntestinal
GLP	Glucagon-Like Peptide
GLUT	Sodium-dependent Glucose Transporter
HLA	Human Leukocyte Antigen
IA-2	Tyrosine-Phosphatase-2
ICA	Islet Cell Antigen
IEC	Intestinal Epithelial Cell
IEL	Intraepithelial Lymphocyte
IFN	Interferon
Ig	Immunoglobulin
IgA	Immunoglobulin A
IL	Interleukin
IL-2R	Interleukin-2 Receptor
ISCA	Islet Cell Surface Antigen
kDa	kiloDalton
KRH	Krebs-Ringer-Hepes
LAB	Lactic Acid Bacteria
LP	Lamina Propria
Mad-CAM	Mucosal Addessin Cell-Adhesion Molecule
MHC	Major Histocompatibility Complex
MLN	Mesenteric Lymph Nodes
NOD	Non-Obese Diabetic Mouse
PBS	Phosphate Buffered Saline
PMA	Phorbol Myristate Acetate
PP	Peyer's Patches
RIP-NP	Rat insulin promoter nucleoprotein
RIP-OVA	Rat insulin promoter OVA
SCFA	Short Chain Fatty Acid
SGLT	Sodium-Independent D-Glucose Transporter
TCR	T-cell Receptor
TGF	Transforming Growth Factor
T_h1	T-helper type 1

T_h2 ,	T-helper type 2
TNF	Tumor Necrosis Factor

Chapter 1: Literature Review

Introduction

It is well established that Type I diabetes, or Insulin Dependent Diabetes Mellitus, is autoimmune in nature, but the pathogenesis has yet to be determined. In autoimmune (type 1) diabetes, cells of the immune system infiltrate the pancreas and destroy the insulin-producing ß-cells in the Islets of Langerhans in an inflammatory process termed insulitis. Certain genes predispose individuals to diabetes, though, discordance in rates of diabetes among genetically identical twins suggests that there is also a strong environmental influence to the development of the disease (Kumar *et al*, 1993;Matsuda & Kuzuya, 1994). It has been suggested that a genetic predisposition in combination with an appropriate environmental trigger could be a significant risk factor; however the precise environmental trigger has not been identified.

In humans, early infant feeding practices and time of weaning have been implicated in the pathogenesis of diabetes (Gerstein, 1994;Kostraba *et al*, 1993). Diet has been shown to modulate expression of diabetes in animal models that are susceptible to the disease, such as the Diabetes-Prone BioBreeding (BB) rat and the Non-Obese-Diabetic (NOD) Mouse (Scott *et al*, 1985) (Elliott *et al*, 1988). This suggests that a mechanism at the level of the gut may be involved in initiating or propagating the events that lead to diabetes. Despite this, investigation targeting the gut in the pathogenesis of diabetes is scarce. Research into the mechanisms of the dietary modulation of diabetes has focused on the macronutrient content of the diet with the exception of fibre, despite evidence that dietary fibre may have an immunomodulatory effect in the gut of healthy individuals (Field *et al*, 1999b). Fibre is an important component in the weaning foods of young children (Agostoni *et al*, 1995) and may therefore be necessary in the normal development of a healthy gastrointestinal tract and gut immune system; however, the specific role of fibre in healthy gastrointestinal development has not really been addressed.

The effect of fibre in the gastrointestinal tract of BBdp rats is unknown, however, it has been suggested that BBdp rats have an abnormal gut response to a nutritionally complex, cereal-based weaning diet such as the standard laboratory chow (Reimer *et al*, 1998). This chapter will describe the BB rat as an animal model of human diabetes and the etiological role of the immune system in its development. As the gut has been implicated in the pathogenesis of diabetes, the gut-associated lymphoid tissue (GALT), its development during weaning will also be addressed. Furthermore, the GALT in animal models of diabetes, and humans and potential of diet to modify

diabetes incidence will be discussed in addition to potential mechanisms by which diet may influence the development of the autoimmune disease. The role of dietary fibres in the gut and GALT will also be discussed.

The BB rat as an animal model of Type 1 Diabetes

The diabetes-prone Biobreeding (BBdp) rat is a unique model of Type 1 Diabetes discovered in Wistar rats in 1970 (Nakhooda *et al*, 1977). BBdp rats can spontaneously develop insulin-dependent Type 1 Diabetes in a manner similar to the human type 1 diabetic syndrome. A diabetes-resistant strain of BB rats, termed normal BB (BBn) rats has subsequently been bred, characterized and has a rate of diabetes of less than 1% (Like *et al*, 1982;Like *et al*, 1991). Diabetes-prone BB rats develop diabetes between 60-100 days of age with a variable incidence rate between 30-90% (Field & Butler, 1999). Unlike the murine model of spontaneous Type 1 diabetes, (the Non-Obese Diabetic (NOD) mouse) (Tochino, 1987), occurrence of diabetes among BB rats is equal between sexes (Bach, 1988). Similar to the human syndrome, disease expression in the BB rat is characterized by insulin deficiency, resulting in the classic clinical symptoms of diabetes such as hyperglycaemia, ketoacidosis, hyperglucagonemia, hyperaminoacidemia, ketonuria, weight loss, and death if not treated (Field & Butler, 1999).

BBdp rats have a profound lymphopenia, which is characterized by low or absent levels of T-cells, affecting most severely the CD8+ population and RT6+ population of T-cells (Elder & Maclaren, 1983) (Yale *et al*, 1985) (Greiner *et al*, 1986). Lymphopenia is a result of a defective lyp gene, independent of the MHC genes (Field C.J & Butler S, 1999). RT6 is a glycosyl-phosphatidyl inositol-linked alloantigen found on more than 70% of peripheral memory T-cells in rats and is also present in high concentrations on intraepthelial lymphocytes (IEL) (Fangmann *et al*, 1991;Rigby *et al*, 1996;Waite *et al*, 1996). . Lymphopenia is not observed in humans (Pontesilli *et al*, 1986).

Lymphocyte metabolism in BBdp rats differs from that of BBn rats (Field *et al*, 1991). Glucose metabolism in unstimulated splenocytes (Field *et al*, 1991) and glucose and glutamine metabolism in unstimulated mesenteric lymph node (MLN) cells (Field *et al*, 1994) were elevated compared to cells from BBn rats. Mitogen stimulation of BBdp immune cells with either Phorbol Myristate Acetate (PMA) and Ionomycin or Concanavalin A (ConA) (Maclaren *et al*, 1983) results in a lower incorporation of ^{3}H-thymidine and a lower production of the cytokine IL-2, a

powerful inducer of T-cell proliferation important in the development of the immune system, when compared to BBn rats (Field & Butler, 1999).

Despite similarities to the human disease, it will be shown that aspects of disease pathogenesis differ substantially among the two animal models of diabetes, the BB rat and the NOD mouse and humans. The clinical relevance of findings from animal models should be approached with caution when applying these principles to the human population (Greiner *et al*, 2001). Despite marked differences between humans and animal models, the BB rat has furthered our understanding of the autoimmune process in diabetes considerably (Greiner *et al*, 2001)

Etiology of Diabetes: Cellular and Humoral Immunity

It is well known that the deletion of autoreactive T-cells during ontogeny occurs in the thymus through negative selection (Anderton & Wraith, 2002). Some autoreactive T-cells may escape the negative selection process, but peripheral immunoregulatory mechanisms maintain a self-tolerant T-cell repertoire. Therefore, the presence of autoreactive cells in the body may be normal and constitutive; however, strict immunoregulatory processes within the body prevent the activation and subsequent clonal expansion of these cells (Anderton & Wraith, 2002). It is thought that in diabetes or other autoimmune conditions, there is a failure of regulation of immune function that results in the inability to suppress autoreactive immune cells upon exposure to a certain self-antigen, or a foreign antigen resembling a self-antigen (molecular mimicry) (Rabinovitch, 1998). Contributors to the proposed dysregulation may include abnormal antigen presenting cells (Marleau & Singh, 2002), abnormal expression and function of costimulatory molecules (Moore & Bellgrau,1999; Delemarre *et al*., 2001) , and abnormal production of regulatory effector molecules, such as cytokines. In the BB rat, there appears to be an absent putative regulatory T-cell population (Greiner *et al*, 1987), activation of immune cells in the absence of a co-stimulatory signal (Moore & Bellgrau, 1999), and decreased production of regulatory cytokines after mitogen stimulation (Field *et al*, 1999a).

In humans, possession of certain Major Histocompatibility Complex (MHC) class II or HLA-DR and DQ alleles is associated with an increase in the risk of diabetes (Notkins, 2002). In BB rats, many of the genes associated with diabetes are also found in the MHC region, bearing the $RT1^u$ haplotype (Field & Butler, 1999). MHC class II genes encode for the expression of intracellular antigen processing and extra cellular presentation molecules (Villadangos, 2001).

Soluble, extra cellular antigen is taken up by the cell, arranged with MHC molecules, and presented to CD4+ T-cells on the surface of the cell (Villadangos, 2001). The role of MHC class II molecules in autoimmunity may be a result of enhanced binding of autoantigens in the MHC, or abnormal MHC expression in the thymic microenvironment during negative selection processes, where autoreactive T-cells would normally be deleted (reviewed by Ridgeway & Fathman, 2000).

In BB rats, lymphopenia, affecting most severely the RT6+ T-cell population, is the most pronounced immune abnormality (Greiner *et al*, 1986). RT6 is expressed on mature CD4+ and CD8+ T-cells (Greiner et al., 1997)(Fangmann *et al*, 1991). Despite absent RT6+ cells in the periphery, intraepithelial lymphocytes (IEL's) in BBdp rats include some RT6+ cells, suggesting an extrathymic origin for these cells(Fangmann *et al*, 1991). Lymphopenia is necessary for diabetes development in the BB rat; however, its presence does not guarantee development of diabetes (Yale *et al*, 1985). The observed lymphopenia in BB rats and relation to development of diabetes, in particular the absence of RT6+ T-cells, suggests a possible regulatory role of this cell-subset (Greiner *et al*, 1987). In one study, adoptive transfer of RT6+ T-cells from BBn rats to BBdp rats prevented diabetes in the BBdp rats (Burstein *et al*, 1989). As well, RT6-depleted BBn rats were more susceptible to diabetes than their RT6+ BBn counterparts (Greiner *et al*, 1987).

Increased lymphocytic metabolic activity and decreased *in vitro* response to mitogens is consistent with the hypothesis that immune cells from BBdp rats are activated *in vivo* prior to mitogen stimulation, or activation (Field *et al*, 1991). More recent findings are in agreement with this hypothesis (Moore *et al*, 2001;Moore & Bellgrau, 1999). Activation, and progression into the cell cycle was observed in the absence of a second costimulatory signal by lymphocytes from BBdp animals (Moore & Bellgrau, 1999), associated with a decrease in the level of a cyclin-dependent kinase inhibitor, p27(kip) (Moore *et al*, 2001).

The Role of Cytokines in Diabetes Pathogenesis

CD4+ T-helper cells (T_h) when activated, differentiate into two subsets (T_h1 and T_h2) that are involved in different aspects of the cell-mediated and humoral immune response. T_h1 cells secrete cytokines that are associated with the inflammatory response, such as Interleukin-2 (IL-2), interferon-γ (IFN-γ), tumour-necrosis-factor-α (TNF-α) (Mosmann, 1991) (Rabinovitch, 1998).

T_h2 cells secrete cytokines (IL-4, IL-5, and IL-10) that are involved in preventing an inflammatory response and stimulate humoral immunity (Mosmann, 1991;Rabinovitch, 1998).

An imbalance in the ratio of T_h1/T_h2 T-cell subsets and the subsequent production of their effector molecules may be contribute to the autoimmune process of diabetes (Rabinovitch *et al*, 1996). In human studies, it has been demonstrated that peripheral lymphocytes, when stimulated with a mitogen, produce more T_h1 cytokines, such as IFN-γ, and less T_h2 cytokines, such as IL-4 and IL-10 (Rapoport *et al*, 1998). A hypersecretion of T_h1 cytokines has also been found in healthy first-degree relatives of patients with diabetes (Hussain *et al*, 1998).

It has been hypothesized that T_h1 cytokines are destructive to pancreatic β-cells, and T_h2 cytokines are protective (Rabinovitch, 1998). Abnormal cytokine production can lead to β-cell destruction either directly, by direct cytotoxicity, or indirectly, through initiating a cascade of events that result in β-cell destruction, by cytotoxic cells (Rabinovitch, 1993). Both in *vitro* and *in vivo* data support this theory(Rabinovitch, 1998). *In vitro*, TNF-α can induce fas expression on β-cells, rendering them more susceptible to cytotoxic lysis by CD8+ T-cells (Amrani *et al*, 2000). Additionally, *in vitro* treatment of β-cells with IFN-γ and IL-1β has been shown to render them more susceptible to immune destruction by CD4+ T-cells (Amrani *et al*, 2000). Direct cytotoxicity to both human and rodent β-cells has been demonstrated when two or more of these cytokines in combination are added to culture (Rabinovitch & Suarez-Pinzon, 1998).

In vivo studies have found that the cytokine gene expression in the infiltrates of the pancreas of BBdp animals is shifted towards T_h1 profile, with increased expression of IFN-γ (Zipris, 1996) (Rabinovitch *et al*, 1996). The role of IFN-γ in the pathogenesis of diabetes was demonstrated by administration of an anti-IFN-γ antibody, which resulted in a decreased incidence of diabetes in the BB rat, decreased activation of immune cells, and insulitis (Nicoletti *et al*, 1997). IL-4 has been shown to reduce the incidence of diabetes when splenocytes transfected with IL-4 were transferred to BBdp animals (Zipris & Karnieli, 2002).

It has been suggested that administration of certain T_h1 cytokines, such as TNF-α or IL-2, can reduce the incidence of diabetes in susceptible animals, as deficiencies in endogenous production could be involved in the dysregulation of immune responses (Rabinovitch & Suarez-Pinzon, 1998). Earlier studies have shown that diabetes in BB rats can be inhibited with TNF-α administration (Satoh *et al*, 1990). Furthermore, El-Sheihk et al., (1998) found that TNF-α, a T_h1 cytokine, expression was positively correlated with that of IL-4, a T_h2 cytokine. Finally, BBdp

rats have demonstrated reduced ability to produce TNF-α by peritoneal macrophages compared to BBn animals (Lapchak *et al*, 1992). These findings suggest a regulatory role for TNF-α in the pathogenesis of diabetes, as opposed to the observed *in vitro* cytotoxic effect. In NOD mice, treatment with IL-2 has shown to reduce incidence of diabetes, perhaps by correcting a pre-existing deficiency (Rabinovitch & Suarez-Pinzon, 1998). BBdp rats also display a reduced ability to produce IL-2 *in vitro* after mitogen stimulation as discussed previously (Field, 1995). However, it was reported that treating a BB rat subline with IL-2 accelerated the development of diabetes (Zielasek *et al*, 1990), suggesting that a pre-existing IL-2 deficiency may not play a role in the pathogenesis.

Controversial in the field of type 1 diabetes is the trigger that initiates the aberrant immune attack on the pancreas. In humans, circulating autoantibodies to islet cell surface antigens (ICSA) before diabetes onset have been identified and can be used as a screening tool to evaluate risk for the development of the disease (Notkins & Lernmark, 2001; Thivolet *et al*, 2002). Islet cell autoantibodies in humans against glutamic acid decarboxylase (GAD), Tyrosine phosphatase IA-2, and insulin have been reported (Notkins & Lernmark, 2001). The appearance of autoantibodies in individuals with diabetes or at high risk for diabetes compared to healthy controls implicates a number of potential autoantigens. Patients with newly diagnosed diabetes have consistently demonstrated increased humoral and cellular immunity to islet-cell autoantigens *in vitro* compared to healthy controls (Durinovic-Bello *et al*, 1996;Hummel *et al*, 1996;Petrovsky *et al*, 2002)

The appearance of autoantibodies in the BB rat against ICSA has been reported (Dyrberg *et al*, 1983;Myers *et al*, 1998;Ziegler *et al*, 1994), however this has not been a consistent finding as other studies have reported autoantibody levels being similar to non-diabetes-prone controls (Mackay *et al*, 1996); De Silva et al., 1996; (Dyrberg *et al*, 1984). Furthermore, it has been demonstrated that the appearance of autoantibodies is not associated with diabetes onset (Schlosser *et al*, 1994; Ziegler *et al*, 1994). Islet cell surface antigens reported in BB rats include a 38 kDa β-cell antigen (Ko *et al*, 1994), glutamic acid decarboxylase (GAD), carboxypeptidase H(Rossini *et al*, 1995), sulfatide (Buschard *et al*, 1993) and Islet cell Antigen (ICA)-512(Myers *et al*, 1998)) . Despite lymphopenia in BBdp animals, B-cell numbers are normal and do not differ in ability to produce immunoglobulin (Ig) from their diabetes-resistant counterparts, BBn rats (Maclaren *et al*, 1983). Autoantibodies have not been shown to transfer the disease in animal models, with T-cells being necessary for disease transfer (Birk & Cohen, 1993). Additionally,

injecting GAD65 (Bieg *et al*, 1999;Petersen *et al*, 1997), or insulin β-chain peptide (Bieg *et al*, 1999) into BBdp animals did not increase the incidence of diabetes suggesting that these may not be significant autoantigens in this particular model. The association between autoantibodies and the pathogenesis of Type 1 diabetes is not clear and remains for elucidation. The triggering autoantigen has not been identified in BB rats, despite the relationship to diabetes of GAD65 and insulin in the NOD mouse and potentially, in humans.

GALT: Role of the Gut in Mucosal Immunity

The gastrointestinal tract is a multi-functional organ responsible for the digestion and absorption of nutrients. In the gut, systemic circulation and the external environment are separated by a single cell layer. The gut is constantly exposed to environmental stimuli, such as foreign food and bacterial antigens. In healthy individuals, immune cells in the gut can maintain a tolerant state, not being activated by food antigen. It is necessary therefore to have a specialized primary defense system that protects against systemic antigen and pathogen entry, while maintaining normal nutrient passage across the epithelium. The primary defense system is known as the Gut-Associated Lymphoid Tissue (GALT). GALT must be able to distinguish food-related antigens and resident bacteria and tolerize the immune system to these agents. Failure to tolerize the immune system to common food antigens may result in allergy or autoimmune disease (Strobel, 2002).

The gut and GALT work together to provide innate (non-specific) and adaptive (specific) immunity to the body. There are three major outcomes to systemic exposure to an oral antigen. Firstly, there is the induction of oral tolerance, secondly, the immune system could be primed against the antigen, and finally, feeding an antigen could result in the formation of sIgA antibodies completely preventing any further exposure to the immune system by the antigen (Strobel, 2002). Innate immunity provided by the gut includes physical barriers between the intestinal lumen and systemic circulation, such as the glycocalyx and mucous lining, and intestinal epithelial layer (Kagnoff, 1993). Additionally, physiological mechanisms such as peristalsis, gastric acidity and bile salts help control exposure of the host to harmful pathogens (Langkamp-Henken *et al*, 1992). In addition to barrier function against the outside environment, GALT provides immune protection throughout the gastrointestinal tract.

GALT is thought to consist of about 25% of the gut mucosa, and also contains 70 % of the body's immunoglobulin-secreting cells, making the GI tract a significant immunological

organ (Wu et al., 1999; Kagnoff, 1993). The GALT is comprised of the mesenteric lymph nodes (MLN), the Peyer's Patches, intraepithelial lymphocytes, lamina propria lymphocytes and various types of antigen-presenting cells (APC) (Mowat & Viney, 1997). In this section, the structure and function of GALT will be discussed, as well as its possible contribution to autoimmune disease.

The mesenteric lymph nodes (MLN) are secondary lymphoid organs that are proximal to the small intestine. They are not directly in the gut, but consist of cells that are entering and leaving the gut (Van Rees *et al.*, 1990). Lymphocytes that have been activated in the Peyer's Patches pass through the MLN to the thoracic duct, and after peripheral circulation, pass through again to the lamina propria (Van Rees *et al.*, 1990).

The Peyer's Patches (PP) are organized lymphoid tissue in the small intestine that are thought to be the principle site of antigen presentation to T and B-cells in the GALT. Peyer's patch lymphocytes are very responsive to gut antigen and give rise to antibody-producing cells in the lamina propria (LP) and to systemic suppressor cells, involved in oral tolerance (Lyscom et al., 1982). The PP consist of lymphoid follicles and interfollicular zones (Kagnoff, 1993). The follicles contain germinal centers and are mainly comprised of B-cells (Kagnoff, 1993). T-cells in the Peyer's Patches are found mainly in the interfollicular zones (Kagnoff, 1993) and include CD4 + T-cells and CD8+ T-cells, associated with helper-inducer function and cytotoxic suppressor functions, respectively (Langkamp-Henken *et al*, 1992). Naïve B-cells are also present within the PP (Kagnoff, 1993).

Each PP is lined with a thin layer of M-cells on the luminal side . M-cells are specialized, highly endocytic cells that are differentiated from normal intestinal epithelial cells by their lack of microvilli (Langkamp-Henken *et al*, 1992). Furthermore, a scarcity of goblet cells in the epithelium overlying the Peyer's Patches results in gaps in the mucus layer allowing the M-cells access to luminal contents (Langkamp-Henken *et al*, 1992). M-cells do not express class II MHC, rather, they endocytose intact antigen which is taken up by resident dendritic cells, macrophages, mature T- and B-cells in the Peyer's Patches for processing and presentation (Mowat & Viney, 1997).

CD4+ T-cells within the Peyer's Patches mediate the suppression of IgM and IgG production by B cells and promote IgA production in a process known as class-switching (Langkamp-Henken *et al*, 1992). Activated IgA-secreting B-cells leave the Peyer's Patches via the mesenteric lymph nodes and enter the systemic circulation through the thoracic duct. They

subsequently migrate to mucosal tissues in the intestine such as the lamina propria to secrete antibody into the lumen (Abreu-Martin & Targan, 1996). T-cells activated in the Peyer's Patches also migrate to intra-epithelial spaces and the LP (Langkamp-Henken *et al*, 1992).

Lamina Propria

The LP region of the intestine is non-organised lymphoid tissue underlying the epithelial layer. Cell phenotypes in the LP are mainly B-cells, and CD4+ T-cells (Kagnoff, 1993). The phenotype of the LP lymphocytes is predominantly activated and memory (expressing the CD45RO form of the CD45 tyrosine phosphatase) T-cells and antibody secreting plasma cells (Kagnoff, 1993). The B-cells present in the LP are mainly IgA secreting plasma cells, but secrete polymeric IgA (Langkamp-Henken *et al*, 1992). Other cell phenotypes present in the LP include macrophages, eosinophils, and mast cells (Langkamp-Henken *et al*, 1992) and dendritic cells (Maric *et al.*, 1996). A major characteristic of the lamina propria is a chronic physiological inflammation which is controlled due to regulatory suppressor mechanisms (Mayer, 2000). This inflammation is in the form of lymphocyte-filled villi and it is thought to play a role in protection of the gut (Mayer, 2000).

Intra-Epithelial Lymphocytes

The intraepithelial Lymphocytes (IEL's) are a unique population of cells that reside in the epithelial layer dispersed among intestinal epithelial cells. IEL's consist mainly of CD8+ T-cells. Many of these CD8+ lymphocytes express a CD8 a-a homodimer in contrast to peripheral CD8+ cells that express the CD8 a-ß heterodimeric form of the CD8 molecule(Kraehenbuhl & Neutra, 1992). To further differentiate IEL's from other lymphocyte populations, IEL's express a β_7 integrin molecule whose ligand on enterocytes is E-cadherin. Ligation of the β_7 integrin and E-cadherin results in repopulation of the epithelial layer by cells formerly activated in the GALT (Abreu-Martin & Targan, 1996). This integrin expression is regulated by enterocyte-derived TGF-ß (Mowat & Viney, 1997).

TCR+αβ IELs are more abundant in the ileum than in the jejunum of rats (Helgeland *et al*, 1997). The same variability was observed in germ-free rats, ruling out the possibility of microbial colonization as a determinant for IEL T-cell populations. This suggests that the expansion of IEL's is not entirely antigen-dependent and that variability could be due to some endogenous factor (Helgeland *et al*, 1997). This stresses the evolutionary role that these cells are

thought to play, a first line of defence, especially during ontogeny, in the mucosa. The specific roles of IEL's are not clearly understood, however, a few hypotheses exist. Is it possible that IEL's kill damaged or virus-infected cells, ensuring mucosal integrity (Kraehenbuhl & Neutra, 1992). The recognition of stress molecules by IEL expressed on epithelial cells suggests a possible role in gut maintenance, and will be discussed further in the next section (Mayer, 2000).

Gamma-Delta T-cells

IEL's contain a T-cell subset that express the ?,d form of the T cell receptor, as opposed to the a,ß, (Salerno & Dieli, 1998). Limited junctional diversity may be important in the types of antigens recognised by ?d T-cells, as the antigens tend to be more conserved and ubiquitous in nature (Abreu-Martin & Targan, 1996). They are mainly CD8+ but express the a,a-homodimeric form of the molecule, similar to other IEL's (Salerno & Dieli, 1998). In mice, they appear earlier than αβ T-cells, suggesting a role in early immune protection for the organism, before acquired immunity develops completely (Hanninen & Harrison, 2000). In the rat intestine, it has been reported that ?d T-cells comprise about 5.6 and 9.4 % of the IEL's in the jejunum and ileum of the rat, respectively (Helgeland *et al*, 1997).

Unlike αβ T-cells, ?d-T-cells are not restricted by their recognition of MHC (Salerno & Dieli, 1998). Typically, CD8+ T-cells recognise antigen in the context of class I MHC, however, special characteristics of the ?d-TCR may enable these cells to recognise other antigen presentation molecules. One molecule recognized by the ?d TCR is the non-polymorphic CD1 antigen expressed on the surface of human and mouse APC. CD1 was shown stimulate secretion of Th1-type cytokines in ?d T-cells (Spada *et al*, 2000). The limited polymorphism of the CD1c molecule allows it to present conserved molecules such as lipid and glycolipid fractions from pathogenic microorganisms (Spada *et al*, 2000). Another cell-surface antigen recognised by ?d-T-cells is the MICA Class I-related antigen (Wu *et al*, 2002). This cell-surface antigen, normally recognised by NK cells, does not present processed peptides like MHC. It is inducible in most cells as a sign of stress (Wu *et al*, 2002). Other non-protein antigens recognized by ?d T-cells include prenyl pyrophosphates and alkylamines which are also products of bacteria, not presented by conventional MHC (Tanaka *et al*, 1995). Evidence suggests that ?d T cells may be extra-thymic in origin in mice, and this has also been found in rats (Ishikawa *et al*, 1999); Ramanathan *et al*, 2002).

Other roles of ?d T-cells include recruitment of inflammatory cells by chemokine secretion, and mediating both acquired and innate immunity through effector molecules as both Th1 and Th2-cytokine producing ?d T cells have been found *in vitro* (Salerno & Dieli, 1998). ?d T cells play a role in ensuring mucosal integrity as mentioned above by the recognition and destruction of stressed or virally infected cells, but as well as regulation of epithelial homeostasis as growth factors affecting epithelial proliferation are also produced (Boismenu & Havran, 1994).

Antigen Presenting Cells

Sampling of antigens in the gut is not limited to the M-cells in the Peyer's Patches. Classical Antigen Presenting Cells (APC) are present in the GALT as macrophages, dendritic cells, mature T- and B-cells and are normally of lymphoid or myeloid lineage, however, non-classical APC exist, such as intestinal epithelial cells (IEC). IEC express class II MHC antigens, which present antigen to mucosal CD4+ T-cells (Mowat & Viney, 1997). IEC also express CD1 in mice in human as mentioned above and may thus play a role in activating ?d T-cells (Mayer, 2000). CD1 has also been shown to be expressed on Paneth cells, differentiated intestinal epithelial cells located in cryptic epithelium, however, other antigen presentation molecules have not yet been found on these cells (Porter *et al*, 2002).

Dentritic cells play an important role in antigen presentation and processing in the gut. In order to achieve optimum antigen exposure, dendritic cells can reach with their projections between epithelial cells into the lumen to endocytose antigen (Rescigno *et al*, 2001) This may explain why specificity for antigens that did not pass the luminal barrier has been observed by Peyer's Patch cells (Rescigno *et al*, 2001). Dendritic cells are able to do this as they possess tight junction proteins so the integrity of the epithelial barrier is not disturbed (Rescigno *et al*, 2001).

Co-Stimulatory Molecules in the Gut

T-cells require the engagement of the T-cell Receptor (TCR) with class I (CD8+ cells) or II (CD4+cells) MHC to become activated (Alegre *et al*, 2001) However, an intrinsic regulatory mechanism provided by the body is that for effective stimulation, there must be two signals present; the TCR/MHC complex, which requires CD4/CD8 molecules, and a second signal, or co-stimulatory signal, B7-1 (CD80) or B7-2 (CD86) on the antigen-presenting cell with ligands CD28 or CTLA-4 on the T-cell (Alegre *et al*, 2001). Other co-stimulatory molecules exist, such

as LFA-3 (CD58), however, this discussion will focus on CD80. Ligation of CD80 with CD28 results in T-cell activation, however, ligation with CTLA-4 results in a down-regulation of the T-cell response. CTLA-4, therefore, plays a key role in immune tolerance. Antigen-presentation in the presence of a costimulatory signal (CTLA-4) is equally as important as TCR/MHC receptor engagement without a costimulatory signal in maintaining peripheral self-tolerance (Sun *et al*, 1999). CTLA-4 has been shown to be a crucial element in the development of oral tolerance (Fowler & Powrie, 2002).

IEC have been shown to lack CD80/CD86 expression (Sanderson *et al*, 1993). This may contribute to tolerance as TCR engagement in the absence of co-stimulation results in T-cell anergy(Alegre *et al*, 2001). Absent costimulatory molecules may prevent the activation of CD4+ T-cells, while favouring the stimulation of suppressive CD8+ IEL's by the CD1 presentation molecule (Strobel, 2002). It has been proposed, that in inflammatory diseases in the gut, that there is aberrant expression of CD80 or CD86 in the gut (Nakazawa *et al*, 1999). One study reported that CD80 is not expressed in the colonic epithelium of healthy patients, nor patients with ulcerative colitis by immunohistochemical analysis (Bloom *et al*, 1995). Another study found that CD86, but not CD80, was found on inflamed colonic mucosa from patients with inflammatory bowel disease (IBD) (Nakazawa *et al*, 1999) and infection with *Helicobacter Pylori* (gastric mucosa) (Ye *et al*, 1997). These studies suggest a role of costimulation in inflammation in the gastrointestinal tract.

Homing of Immune Cells Activated in the Gut

A unique property of the GALT is the ability of lymphocytes activated in the MLN and PP to recalculate back to mucosal tissues (Mowat & Viney, 1997). IEL's injected into SCID mice repopulate the intestinal epithelium (Sydora *et al*, 1993). High endothelial venules in the Peyer's Patches, MLN and gut mucosa endothelium contain vascular addressin cell adhesion molecules specific to mucosa called MAdCAM-1(Abreu-Martin & Targan, 1996). The ligand for MAdCAM-1 is an integrin $\alpha_4\beta_7$ expressed specifically on activated memory T-cells posessing the CD45RO isoform of the tyrosine phosphatase CD45 (Schweighoffer *et al*, 1993). It has been reported that T-cells expressing the $\alpha_4\beta_7$ integrin do not express high levels of $\alpha_4\beta_1$ integrins, involved in other site-specific homing, indicating that the $\alpha_4\beta_7$ is a gut-specific homing molecule (Schweighoffer *et al*, 1993). β_7 Integrin knock-out mice do not have lymphocytes in their Peyer's

Patches, lamina propria, or epithelium (Wagner *et al*, 1996). Gut-homing has been demonstrated in humans as well as rodents (Farstad *et al*, 1996), enabling cells activated in the gut by luminal antigens to perform effector functions in the region where they will most likely re-encounter the antigen.

Humoral Mucosal Immunity

Secretory IgA (sIgA) is the main immunoglobulin secreted in the intestine and is responsible for most of the antibody responses in the mucosal tissues (Langkamp-Henken *et al*, 1992). It has been proposed that sIgA is a first line of defence in the GALT as the secretory component (SC) (part of the sIgA) appears in human newborns after 1 week of birth at levels similar to adults (Rognum *et al*, 1992). IgA secretion occurs after a B-cell is exposed to antigen in the Peyer's patch and is activated by a CD4+ T- cell. Secretion of Th2 cytokines by CD4+ T-cells lead to differentiation from a B-cell to an Ig-secreting plasma cell (Mega *et al*, 1992). T-cells within the PP induce a class switch to IgA producing cells by secreting TGF-β (Mayer, 2000). Therefore CD4+ T-cells are necessary for sIgA production in the intestine (Mega *et al*, 1992).

IgA secreted in mucosal tissues differs from that present in the peripheral circulation. In the serum, IgA is present as a monomer, but in the intestine, it is secreted as a dimer, composed of two monomer units bound by a protein called the J-chain (Langkamp-Henken *et al*, 1992). Secretory component, which originates as a transmembrane protein located on the basal and luminal side of intestinal epithelial cells called the polymeric immunoglobulin receptor, binds to IgA and J-chain (Kagnoff, 1993). Secretory component is also necessary to prevent degradation of IgA by luminal proteases (Mayer, 2000). Membrane-bound IgA is taken up by the cell and transported to the apical surface of the cell where it is then secreted into the lumen as sIgA (Langkamp-Henken *et al*, 1992). Luminal IgA is either excreted, or reabsorbed in the distal ileum, travelling to the portal vein and into the liver where it is taken up by Kueppfer cells which destroy any bound microorganisms, releasing free IgA into the bile (Mayer, 2000).

Due to the structure of sIgA, it does not fix complement or kill bacteria. This is beneficial in that it does not induce mucosal inflammation (Langkamp-Henken *et al*, 1992). IgA appears to bind to luminal pathogens and proteins, preventing the attachment of microorganisms to the epithelial surface, or exposure of the immune system to foreign proteins (Kagnoff, 1993). The importance of IgA is demonstrated by IgA-deficient individuals. People with IgA deficiency

are at increased risk of autoimmune disorders (Cunningham-Rundles *et al*, 1978). Additionally, sinopulmonary infections, allergic reactions and GI complaints are frequently reported (Cunningham-Rundles *et al*, 1978).

Dietary Influence on GALT

It is well-documented that dietary intake can influence the immune system (reviewed by (Field *et al*, 2002). However, diet also has a local effect on GALT. For example, protein-deficient rats have lower absolute amounts of IEL's (Maffei *et al*, 1980). Also, TPN and IV+ intragastic feeding to mice reduced gut IL-4 levels compared to chow and complex enteral diet. IL-10 was reduced in mice treated with TPN only (Wu *et al*, 1999). Other cytokines were not affected by route of nutrition (Wu *et al*, 1999). These studies that show that feeding an antigen can result in systemic hyporesponsiveness implies that diet can profoundly affect the functioning of the immune system.

Oral tolerance

The important role of dietary influences in GALT is demonstrated by the concept of oral tolerance. Oral tolerance is a state of peripheral tolerance to an antigen that was previously administered by the oral route (Strobel, 2002). The evolution of oral tolerance was necessary to prevent hypersensitivity reactions to ingested food and bacterial antigens (Weiner, 2000). Oral tolerance is a form of mucosally-induced tolerance, as peripheral tolerance can also be induced after nasal and bronchial administration of an antigen (Krause *et al*, 2000). There are three main mechanisms by which oral tolerance can be induced. These include active suppression, clonal deletion and clonal anergy. Mechanisms for the induction of oral tolerance depend on dose, nature of antigen, age, and frequency of exposure(Strobel, 1992).

Low, repeated doses of antigen stimulate antigen-specific active cellular suppression of the immune system (Krause *et al*, 2000). It is thought that Peyer's Patches are actively involved in this process as the M-cells endocytose the antigen and expose it to dendritic cells and B-cells which process and present the antigen to CD4+ T-cells (Krause *et al*, 2000). Conversely, oral tolerance has been induced in TNF/lymphotoxin knockout mice, who do not develop Peyer's Patches or MLN (Spahn *et al*, 2002). T_h–cells, once activated, secrete T_h2 cytokines IL-4 and IL-10, and the T_h3 cytokine TGF-β (Krause *et al*, 2000). It has been reported that both IL-4 and IL-10 are required for the induction of active suppression as mice lacking the ability to produce either of these cytokines failed to develop tolerance by this mechanism (Rizzo *et al*, 1999). TGF-

β serves an important function not only in immunosuppression, but in the class switching of B-cells from IgM-producing cells to IgA producing cells (Mayer, 2000). As these cells migrate to the LP region of the small intestine, it ensures that systemic exposure to the particular antigen will be minimal (Krause *et al*, 2000). More recently, γδ T-cells are being recognised as important regulators of oral tolerance. γδ T-cell deficient mice were unable to be tolerized to low dose ovalbumin (OVA) feeding, suggesting a role for γδ T-cells in active suppression (Fujihashi *et al*, 1999). Co-stimulatory molecules are important for the induction of active suppression; blocking CTLA-4 resulted in a failure to develop oral tolerance after low-dose feedings of OVA (Barone *et al*, 2002).

Bystander suppression is another form of active suppression, however, suppression may not be antigen specific, as tolerization may occur as a result of a suppressive immune response to another antigen (Miller *et al*, 1991b). This form of suppression was identified with oral tolerance induction to myelin basic protein, an autoantigen in the rodent model of autoimmune encephalomyelitis (Miller *et al*, 1991b). It would an ideal mode of treatment, which will be discussed below in relation to diabetes, if the two antigens in question are linked anatomically (Mowat & Viney, 1997). TGF-β secreting T-cells may be activated in an antigen-specific fashion, however, migration to a site of an immune response may lead to the suppression of another unrelated response to a different antigen. This could lead to the suppression of an autoimmune response and could have implications in autoimmune disease where the causative antigen is not known. If the initiating antigen were identified, it might then be possible to tolerize cells at the level of the gut to an antigen proximal to the target organ where they would secrete immunosuppressive cytokines (Krause *et al*, 2000).

Clonal deletion and anergy can occur when antigen is fed in high doses (Krause *et al*, 2000). Apoptosis of T-cells in response to high dose antigen feeding has been reported (Krause *et al*, 2000). High antigen dose feeding can also down regulate TCR on T-cells that is specific for the antigen in question (Benson *et al*, 2000). It has been suggested that the high antigen doses fed in studies supporting clonal deletion are not physiologically relevant, suggesting a relatively minor role for this mechanism in oral tolerance induction (Strobel, 2002).

Clonal anergy will occur if antigen presentation occurs in the absence of co-stimulatory signals such as the B7/CD28-CTLA-4 interaction, however, more recent studies favour the role of CTLA-4 in active suppression (Strobel, 2002). Large doses of administered antigen also suppress the induction of regulatory cells, but favourT-cell inactivation (Garside *et al*, 1995)

Neonatal Immune and Gastrointestinal Development

It is important to understand the development of immunity in the neonatal and post-weaning period when considering the potential contribution of early diet to autoimmunity. During gestation and early life, thymic selection occurs and the host T-cell repertoire is being generated. During this time, thymocytes are undergoing positive and negative selection (Anderton & Wraith, 2002). Negative selection ensures that the lymphocytes do not bind too tightly to self-MHC, as that would result in generation of autoimmunity (Anderton & Wraith, 2002). T-cells in the gut may not originate from the thymus, but from cryptopatches in the gut (Ishikawa *et al*, 1999;Poussier & Julius, 1999). Selection processes in these putative cryptopatches have not been as clearly defined as thymic selection processes (Poussier & Julius, 1999).

Neonatal immune function has been shown to be deficient in mice and humans (Adkins, 1999). In neonates, effector function is geared towards a T_h2 response, and under *in vivo* conditions, cannot effectively establish a T_h1 response (Adkins, 1999). A T_h2 bias in pregnancy allows the fetus to survive (Bjorksten, 1999). Administration of an adjuvant *in vivo*, increasing co-stimulatory activity, and endogenous APC-independent stimulation *in vitro* improves indices of T_h1 responses in neonatal T-cells (Adkins, 1999). Neonatal T-cells have an immature phenotype, with high expression of CD45RA, lower proliferative responses and lower production of IFN-γ and IL-4 (Cummins & Thompson, 1997). As the infant ages, the T_h2 immunological phenotype shifts towards the adult T_h1 profile, with a balanced T_h1/T_h2 profile is often associated with maturation (Bjorksten, 1999).

Weaning, which is when the animal switches from suckling to an adult diet, could be considered a challenge to the gut in a normal healthy animal. At weaning, replacing breast-milk with other foods increases the amount of antigen exposure to GALT (Strobel, 1996). Neonatal rats begin to sample maternal diet at around the age of 12-13 days (Herbst & Sunshine, 1969). Weaning is thought to play an important role in intestinal development, as opposed to temporal factors, since early weaning (17 days) is associated with an early maturation of the gastrointestinal tract, characterized by increased intestinal length, decreased lactase activity, and an early increase in sucrase activity compared to 25-day old control weanlings(Lebenthal & Lee, 1983).

Breast-milk is higher in fat, lower in carbohydrate and antigenic content, and contains immunomodulatory factors, such as cytokines TGF-β and sIgA (Hanson *et al*, 1994;Strobel, 1996). As a healthy animal adapts to the change in diet at weaning, there are reports of increases

in the brush border and luminal enzymes (Cummins *et al*, 1991) Changes in morphology, such as increased crypt depth and villus height, occur to provide an increased absorptive capacity (Cummins *et al*, 1988b). Gut maturation at weaning appears closely related to intestinal immune activation. For example, blocking the IL-2 receptor (IL-2R) results in a decreased growth of the intestine during weaning (Thompson *et al*, 1996). Furthermore, mucosal mast cell activation is associated with weaning (Cummins *et al*, 1988a). Interleukin-2 receptor expression peaked at 21 days of age in the MLN cells, Peyer's Patches, lamina propria, and IEL populations at a time that luminal antigens are introduced (Masjedi *et al*, 1999). From birth to to age 20 and 22 days, the MLN weight was reported to increase seven-fold (Cummins *et al*, 1988b). Jejunal eosinophils increase 3 fold (Cummins *et al*, 1988b). Jejunal count of IEL's increase after weaning, from day 22-24 of life (Cummins *et al*, 1988b). MHC class II expression on enterocytes in neonatal animals is low at one week of age but increases at weaning in rats (Strobel, 1996). At weaning it was found that it was more difficult to induce oral tolerance (Strobel, 1996). These data suggest that weaning is associated with immune activation, consistent with the idea that the gut is being immunologically challenged at weaning by the switch from maternal milk to adult diet.

One of the mechanisms by which the gut and GALT mature at weaning could be the increase in food and bacterial antigen exposure (Bjorksten, 1999). Development of mucosal IgA function is dependent on stimulation by resident bacteria (Umesaki & Setoyama, 2000), since the intestinal microflora from 1–23-day-old animals does not stimulate IgA production, while that from a 25-day-old mouse is able to induce intestinal IgA (Umesaki & Setoyama, 2000). This indicates an important role of weaning in defining the intestinal microflora between 23 and 25 days of age in the rat (Umesaki & Setoyama, 2000). It is suggested that the change in the microflora contributes to the change from a T_h2-skewed immune response towards a T_h1 response (Bjorksten, 1999). In conclusion, the immune response in the neonate is immature and biased towards a T_h2 response that shift towards a more balanced T_h1/T_h2 response as the infant encounters antigens, most notably in the gastrointestinal tract. Weaning is associated with gut and gut immune maturation, as a switch from a breast-milk diet to weaning diet results in a number of changes in the gastrointestinal tract.

The gut and Type 1 diabetes

The ability of diet to modulate disease expression in both BB rat and NOD mouse models of spontaneous autoimmune diabetes, as well as some epidemiological human data points to a

pathogenesis that involves the gastrointestinal tract. Few studies have examined whether intrinsic abnormalities in gut and gut immune structure and function could possibly contribute to the pathogenesis. This section will review the studies that have implicated the gut and gut immune system in the development of Type 1 diabetes.

Intestinal permeability to dietary macromolecules has been implicated in systemic antigen exposure (Picco *et al*, 2000). Increased intestinal permeability is observed prior to the onset of autoimmune disorders such as Caeliac's disease and has been observed in a subgroup of patients with Multiple Sclerosis (Johnston *et al*, 2002;Yacyshyn *et al*, 1996). Dietary macromolecules have the potential to initiate an immune response should they bypass intestinal epithelium and gain access to peripheral circulation. There are few studies that have investigated the possibility that intestinal permeability could be involved in the pathogenesis of type 1 diabetes. Existing studies have looked at altered intestinal function, such as increased permeability, as a consequence of the secondary complications of diabetes, such as microangiopathy and neuropathy (Carratu *et al*, 1999).

It has been shown that intestinal permeability to mannitol was higher in patients with type 1 diabetes (showing no sign of Celiac's disease), compared to age-matched controls (Carratu *et al*, 1999). Additionally, permeability was higher to mannitol in a subgroup of children with Type 1 diabetes who possessed the HLA-DQB 1*02 allele, associated with Celiac's disease (Kuitunen *et al*, 2002). However, it is not known if this permeability is involved in the disease process, or is a result of the disease process as measurements were not taken during the prediabetes period. There is evidence that increased gastrointestinal permeability is present before the onset of diabetes in the BB rat. Diabetes-prone BB rats and diabetes-resistant control rats were weaned at 21 days to either chow diet or a hydrolysed casein-based diet. BBdp rats fed either diet showed a greater permeability to sucrose and lactulose/mannitol in the stomach and small intestine respectively. In agreement with these findings, it was reported that BBdp animals had an increase in intestinal inflammatory activity, as demonstrated by higher myeloperoxidase activity in the intestine (Hardin *et al*, 2002)

Gut homing molecules, as described, have been implicated in diabetes in both humans and the NOD mouse. In humans, gut immunohistochemical analysis revealed higher positive staining for $\alpha_4\beta_7$ integrin in the lamina propria (LP) of people with diabetes compared to controls (Savilahti *et al*, 1999). As the $\alpha_4\beta_7$ is found mainly in activated/memory cell phenotypes, this suggests an increase in these cell subsets in the LP of individuals with diabetes (Schweighoffer *et*

al, 1993). Furthermore, GAD65-reactive lymphocytes from individuals with Type 1 diabetes express an increased level $\alpha_4\beta_7$ integrin compared to other homing molecules, suggesting that these autoreactive cells are first activated in the gut (Paronen *et al*, 1997). Human studies did not look at the role of gut-homing molecules in the initiation of diabetes, making their role in the pathogenesis merely suggestive.

The function of gut-homing molecules during the prediabetes period has been investigated in NOD mice. NOD mice had MAdCAM-1 appear on islet endothelium simultaneously to infiltration of lymphocytes and infiltrates expressed $\alpha_4\beta_7$ integrin (Hanninen *et al*, 1993). Additionally, blocking MadCAM-1 with a mAb starting at 3 weeks of life reduced the incidence of diabetes in NOD mice. Interestingly, adoptive transfer of diabetogenic T-cells into NOD/SCID mice when MAdCAM-1 was blocked did not prevent the development of diabetes. Therefore, it is possible that MAdCAM-1 could be involved in initial priming of autoreactive lymphocytes in the gut but does not seem to be necessary for subsequent entry into the pancreas (Hanninen *et al*, 1998).

Evidence of immune activation in the gut has been demonstrated immunohistochemically by increased positive staining of HLA class II -DP and -DR antigens in the villus epithelium and crypts of human subjects with diabetes (Savilahti *et al*, 1999). Staining with antibodies to $\alpha\beta$,$\gamma\delta$ T-cells revealed no differences between healthy controls and patients with diabetes (Savilahti *et al*, 1999). Histological examination failed to detect a difference in intestinal morphology between patients with diabetes and normal age-matched controls (Savilahti *et al*, 1999). These findings suggest some degree of inflammation in the small intestine of patients with diabetes despite normal structure (Savilahti *et al*, 1999). The potential role of increased immune activation before diabetes onset is not known, as several factors could contribute to increased activation post-diagnosis.

The contribution of GALT to the development of diabetes in the BBdp rat has not been well-documented. IEL's from BBdp animals have some RT6+ cells despite an absence of these cells in the periphery (Fangmann *et al*, 1991). However, the cells are significantly reduced in number compared to BBn rat IEL's. It was also shown that the majority of BBdp IEL's were CD4+, as opposed to normal CD8+ IEL's (Fangmann *et al*, 1991). It was demonstrated that BBdp rats have an undetectable amount of ?d T cells in their intestinal epithelium compared to BBn rats (Ramanathan *et al*, 2002). This may explain increased intestinal permeability (Meddings *et al*, 1999) as ?d T cells secrete growth factors involved in epithelial cell homeostasis

(Boismenu & Havran, 1994). Although IEL's, thought to be extrathymic in origin, are present in the mucosa of BBdp animals, their levels are reduced and phenotypes altered compared to BBn rats. As described earlier, ?d T cells play an important role in the regulation of immune responses in the gut. It is possible that the BBdp phenotype could contribute to dysregulation of immune responses to food components in the gut before the initiation of diabetes.

Dietary modulation of Diabetes: Oral Tolerance

Oral tolerance has been suggested as a method of preventing autoimmune disease in animal models. If the antigen that triggers autoimmune attack is known, then it can be fed to prevent autoimmune attack of self-tissues (Krause et al., 2000). In animal models, oral tolerance has been used to prevent the development of experimental autoimmune encephalomyelitis, (animal model of multiple sclerosis), and arthritis (Knoerzer *et al*, 1995). In both cases the initiating antigens are known (Miller *et al*, 1991a). In the case of Diabetes, the antigenic trigger (if only one) is not known, and feeding the same antigens to different animal models has yielded conflicting results.

In NOD mice, feeding oral insulin and GAD65 decreases the incidence of diabetes (Maron *et al*, 2001). Aerosol insulin (subject to tolerization by nasal and lung mucosal tissue) also reduced diabetes in NOD mice, and was associated with an increase in IL-4 and IL-10 production from splenocytes (Harrison *et al*, 1996). Additionally, it was shown that spleen ?d T cells from the above tolerized animals suppressed adoptive transfer of diabetes from diabetic NOD mice to healthy recipients, indicating a key role for ?d T cells in mucosal tolerance, while not specifically oral tolerance (Harrison *et al*, 1996). Age of tolerization may have an effect on the efficacy of insulin feeding in the NOD mouse model, as neonatal feeding was more effective than feeding at 3-4 weeks of age in preventing diabetes (Maron *et al*, 2001).

Oral Tolerance has been shown to prevent diabetes in a transgenic strain of mice that expresses a viral nucleoprotein of the lymphocytic choriomeningitis virus (LCMV) under the control of the rat insulin promoter (RIP) in the pancreatic beta cells (termed RIP-NP transgenic mice) (von Herrath, 1997). Challenge with LCMV virus induces diabetes in 95% of these animals, while is less than 2 % in saline-administered control animals. Insulin feeding shortly before or after LCMV infection prevented virally-induced diabetes in 50% of the animals (von Herrath *et al*, 1996). Bystander suppression was the suggested mechanism in this model as there was still pancreatic infiltration by lymphocytes, however, infiltrates secreted T_h2 cytokines while

control infiltrates secreted T_h1 cytokines (von Herrath *et al*, 1996). Therefore, cytotoxic lymphocytes specific for LCMV virus were still observed in the pancreas, however, suppressor lymphocytes specific for insulin were also present releasing suppressive cytokines that protected the β-cell from immune destruction, providing evidence for bystander suppression in the event of anatomically-linked antigens (von Herrath *et al*, 1996).

Evidence that oral tolerance may be a successful mode of preventing diabetes in animal models is strong, but caution should be used when considering this as a prophylactic treatment in the genetically susceptible human population. It has been found that while some antigens implicated in diabetes could promote oral tolerance in one animal model, it may lead to sensitization in another animal model. This is exemplified by an experimental model using a strain of transgenic mice (Blanas *et al*, 1996). A bone-marrow chimeric RIP-OVA mouse (with bone marrow from another transgenic strain) was used so that CD8+ T-cells would be generated against OVA. When fed OVA, 44% of the transgenic mice developed diabetes compared to controls, suggesting a population of autoreactive gut-derived CD8+ T-cells that could be activated by autoantigen feeding (Blanas *et al*, 1996).

Induction of tolerance in BB rats has been unsuccessful with insulin feeding (Mordes *et al*, 1996). Similarly, feeding BB rats human thyroglobulin did not affect the incidence of thyroiditis (Colzani *et al*, 1999), indicating that there is a general defect in oral tolerance in these animals that is not specific to diabetes. Failure to tolerize the BB rat could be partially explained by their apparent lack of γδ T-cells and IEL in the intestine (Ramanathan *et al*, 2002) , as these subsets have been shown to be important in oral tolerance (Fujihashi *et al*, 1999).

As discussed earlier, age of tolerization could have an impact on oral tolerance induction. However, as the antigen triggering autoimmunity in diabetes remains elusive, Scott et al., (2002) proposed that tolerization to an antigen mixture (such as diabetes-promoting chow) might be a better vehicle for tolerance induction as opposed to a single antigen. Exposing BBdp pups between 4-7 days to a mixture of chow with a hydrolysed casein-based diet significantly delayed diabetes onset and prevented diabetes in one third of the animals weaned to chow (Scott *et al*, 2002). Similarly, neonatal exposure to wheat gluten also had a protective effect when animals were weaned to a diet containing wheat gluten (Scott *et al*, 2002). In BBdp neonates, a decrease in IFN-γ mRNA was observed in the gut with exposure to chow diet in the first week of life (Scott *et al*, 2002). In contrast to neonatal oral tolerance induction, chronic oral antigen administration (with chow) did not result in systemic tolerance induction in BBdp animals, but

did in BBn animals (Scott *et al*, 2002). This suggests that there is a brief period during which BBdp animals can be tolerized, but are unable to develop tolerance with chronic administration.

Treatment of susceptible human populations with oral insulin feeding has been proposed (Pozzilli & Gisella, 2000). In a large multi-national trial (Diabetes Prevention Trial-1), newly-diagnosed subjects were fed 5 g of insulin a day for a year. Feeding insulin did not result in any effect on residual β-cell function, as measured by C-peptide values (Pozzilli & Gisella, 2000). However, oral tolerance therapy at a time when the immune system has already been exposed to the offending antigen, or has been primed, has been unsuccessful in a murine model (Leishman *et al*, 2000). Mice were resistant to tolerance induction after feeding OVA in an OVA- primed immune system. The authors proposed that activated/memory T-cells may be resistant to tolerance or the microenvironment of the primed immune system may inhibit the delivery of tolerogenic signals (Leishman *et al*, 2000).

The role of the gut in the pathogenesis of diabetes is unclear. The gut is the first immune organ to encounter ingested antigen and the way the gut handles the antigen controls systemic exposure. Several studies have implicated the gut and cells of GALT in the pathogenesis, however, few studies have actually addressed the prediabetes period, where cells of GALT may be inappropriately activated. Feeding potential autoantigens may be a mode of prevention of diabetes by inducing suppressor mechanisms in GALT that lead to a β-cell tolerization, and subsequent immune suppression against self. However, a single auto antigen responsible for diabetes has not been identified, and feeding the putative autoantigens has led to unpredicatable types and levels of tolerization in different animal models. However, recent success in preventing autoimmune relapse in patients with multiple sclerosis gives hope that oral tolerance methods may be effective in preventing diabetes in the human population.

Early Diet and Development of Diabetes

The epidemiological evidence surrounding the role of early infant diet and subsequent development of diabetes is controversial at best. Epidemiological data has suggested that short duration of breast-feeding and early introduction of solid foods and cow's milk is associated with an increased risk of developing type 1 diabetes (Borch-Johnsen *et al*, 1984) (Borch-Johnsen *et al*, 1984;Dahl-Jorgensen *et al*, 1991;Kostraba *et al*, 1992) (Kostraba *et al*, 1993) (Fava *et al*, 1994) (Kimpimaki *et al*, 2001). A meta-analysis determined that individuals that developed diabetes were more likely to have been breast-fed for less than 3 months (Gerstein, 1994).

In contrast to earlier findings, another meta-analysis showed that there was only a weak association between early infant diet and subsequent diabetes risk (Norris & Scott, 1996). Recent studies have reported that there is no association between breast-feeding duration and introduction of cow's milk and islet autoimmunity early in life (Couper *et al*, 1999) (Hummel *et al*, 2000) These findings weaken the argument for both cow's milk feeding and short term duration of breast-feeding in the pathogenesis of diabetes. However, they do not completely exclude the concept of early weaning and subsequent development of diabetes in susceptible individuals, as it may not be a component of commercial infant formula that is the diabetogen. Recently, there has been a renewed interest in the potential role of weaning foods, such as wheat-based cereals, in the initiation of Type I diabetes (Beales *et al*, 2002;MacFarlane *et al*, 2003).

How breast milk might have a protective effect, or merely a neutral effect with respect to the development of diabetes remains unclear. It is possible that breast-feeding may delay exposure of the infant to foreign antigens, before maturation of the gut and immune system is complete. However, breast milk itself is a rich antigenic source, as antigens in the maternal diet can be passed through the breast milk to the neonate (Jakobsson, 1991). However, these antigens are delivered with protective factors (discussed below) (Hanson *et al*, 1994), which have the potential to tolerize the infant to some food and bacterial antigens at a time where gut immaturity is still present (Adkins, 1999). The antigens present in breast milk are present at a dose that would favour active tolerance induction (Strobel, 2002).

As previously discussed, the gut and gut immune system in the neonate is functionally immature. Breast-milk provides passive immunity transferred from mother to child (Hanson *et al*, 1994). Some immunological factors transferred in breast milk include secretory IgA, cytokines (Bottcher *et al*, 2000), IgG and IgM antibodies, lysozyme, lactoferrin, and activated/memory T-cells (Bertotto *et al*, 1991;Wirt *et al*, 1992). Secretory IgA, IgG, IgM and activated memory T-cells in the human milk all transfer to the infant immunological memory while the immune system in the infant matures and gains exposure to food and bacterial antigens (Hanson *et al*, 1994). Furthermore, the presence of sIgA specific for a number of bacteria present in the gut is indicative of B-cells that are activated in the gut differentiate and home to the mammary gland where they secrete sIgA (Hanson *et al*, 1994). Breast milk contains analogues of receptors present on intestinal epithelial cells (oligosaccharides) to which bacteria can adhere to establish infection (Hanson *et al*, 1994).

It has been proposed that early exposure to solid foods before "gut closure" may contribute to autoimmune disease. Antigen may be able to cross epithelial layer in the gut before tight junctions close during development, or alternatively, the individual may be exposed to the antigen during an infection (Kostraba *et al*, 1993). In infants fed cow's milk formula, gut closure has been shown to be delayed. It was reported that infants fed cow's milk formula, compared to infants who were breast-fed, in the first week of life did not show a temporal decline in permeability to lactulose, indicating delayed closure of tight junctions in the intestinal epithelium (Weaver *et al*, 1987). Additionally, it has been found that pre-weaned rats have an increased intestinal transport of macromolecules across the epithelium upon exposure to dietary antigens (Isolauri *et al*, 1993).

Breast-feeding and formula feeding yield differing bacterial colonization of the gut after birth, (Hanson *et al*, 1994) At birth, the neonatal gut is colonized by bacteria. Breast-fed infants possess microflora consisting mainly of lactic acid bacteria (gram-positive), while formula-fed infants have a broader spectrum of bacterial species present (gram-positive and negative)(Harmsen *et al*, 2000). Formula feeding can promote the growth of gram-negative bacteria in the intestine, which have the potential to produce endotoxins, which could increase intestinal permeability (Urao *et al*, 1997). Prebiotics, naturally present in breast-milk, are substances that favour the growth of beneficial bacteria (Walker, 2000) Bifidus factor, a prebiotic, stimulates the growth of bifidobacteria (Walker, 2000). This produces a more acidic environment in the lumen, as bifidobacteria produce short-chain fatty acids. In addition, oligosaccharides in breast-milk compete for bacterial receptors on intestinal epithelial cells (IEC). This results in a reduced colonization of the gut with pathogenic flora (Walker, 2000).

Cow's Milk and the "Mimicry Hypothesis"

Earlier studies implicating Cow's milk exposure in early infancy proposed that the autoimmune process was triggered by a structural (amino acid sequence) similarity between a peptide fragment of Bovine Serum Albumin (BSA) in cow's milk called the ABBOS peptide and an Islet cell antigen 69 (Karjalainen *et al*, 1992). In this study, 100% of newly diagnosed diabetic patients were found to have antibodies to BSA (Karjalainen *et al*, 1992). Another study demonstrated that T-cells from children with type 1diabetes were sensitized to bovine serum albumin (Cheung et al., 1994). However, this has not been replicated in other experiments,

suggesting that there may be another antigen involved if indeed, type 1 diabetes is initiated by a "molecular mimicry" reaction to one or more food proteins (Ivarsson *et al*, 1995).

Diet as a Modulator of Diabetes in Animal Models

Diet is a major contributor to the outcome of Diabetes in the BB rat and NOD mouse. It is well-documented that feeding a defined, semi-purified, casein-based diet or an elemental diet can reduce the incidence of diabetes in BBdp rats (Scott *et al*, 1985) (Hoorfar *et al*, 1991), (Issa-Chergui *et al*, 1988;Scott & Marliss, 1991) and NOD mice (Elliott *et al*, 1988) compared to a nutritionally complex , cereal-based chow diet. Based on this, chow diets have been classified as being permissive, or diabetogenic (producing a high incidence of diabetes) or protective (decreasing the rate of diabetes and delaying onset of the disease) (Field *et al*, 1999a). The semi-purified casein-based diets and the chow diets differ considerably in their macronutrient composition (Field, 1995). Chow is higher in carbohydrate (CHO) and lower in fat, than the semi-purified diet, and is less energy dense as energy is provided by CHO, and differs in the composition of fibre, containing fermentable fibres (McBurney et al., 1989). Additionally, compared to the semi-purified casein-based diet, chow contains a mixture of plant and other animal-based proteins, indicative of a higher antigenic load in the GI tract..

Protein composition of the semi-purified diets has been shown to be the most effective modulator of diabetes in BB rats (Scott, 1994). Proteins from wheat, soy and cow's milk proteins have been shown to be diabetogenic (Daneman *et al*, 1987) (Scott *et al*, 1997). Semi-purified, defined diet containing soy protein produced higher incidence than casein-based diet (Hoorfar *et al*, 1991). Other plant proteins considered from chow include wheat germ, and alfalfa seeds both which resulted in intermediate incidence of diabetes (Hoorfar *et al*, 1991), whereas wheat flour and crude soybean meal led to higher incidence than casein-based in NOD mice (Hoorfar *et al*, 1993). It was found that raw red lentils substituted as protein source increased incidence to higher than chow (Hoorfar *et al*, 1992). Lectins present in raw red lentils were hypothesized to be the main contributors, as lectins are known lymphocyte mitogens and may also cause intestinal damage (Hoorfar *et al*, 1992). As cow's milk-based infant formulas have been suggested to be diabetogenic in the human population, there has been attention directed towards its effects in BB rats. However, cow's milk produced variable rates of the disease in BB rats (Malkani *et al*, 1997).

The diabetogenicity of wheat-based diets have more recently been addressed. People with diabetes are more likely to develop gluten-sensitive enteropathy, or Celiac's Disease (Sjoberg *et al*, 1998). Diets in which wheat gluten was the only protein source produced high incidences of diabetes in BB rats (Scott, 1996). In a large, multi-centred trial with BBdp rats and NOD mice, it was found that a milk-free, wheat-based diet produced the highest incidence of diabetes in both rodent strains (Beales *et al*, 2002). Compared to feeding a hydrolysed casein-based diet, feeding a diet with wheat gluten as the sole source of protein resulted in an incidence rate 3 times that of the hydrolysed casein diet in BB rats (MacFarlane *et al*, 2003). Additionally, a specific protein isolated from wheat, a storage protein, G1b1, is associated with pancreatic inflammation and islet damage in BB rats (MacFarlane *et al*, 2003). Antibodies to this protein were found in subjects with diabetes, but not age and sex-matched controls (MacFarlane *et al*, 2003).

Substituting n-3- rich fish oils in the casein-based diet did not affect diabetes incidence compared to the standard semi-purified casein based diet in a number of studies (Hoorfar *et al*, 1992;Issa-Chergui *et al*, 1988) (Kleemann *et al*, 1998). Similarly, changing the fat composition with different oils, such as 17% soybean oil, or 15% safflower oil did not affect the incidence (Issa-Chergui *et al*, 1988) (Hoorfar *et al*, 1992). More work is needed with dietary fats, as n-3 polyunsaturates have been shown to have immunomodulatory effects in BBdp rats, despite no effect on disease incidence (Kleemann *et al*, 1998). The carbohydrate content of the chow diet has not been well-studied in terms of diabetogenicity in BB rats. Dietary fibre has been suggested as a potentiator of diabetes, as the chow and the semi-purified diet both contain different fibre compositions (Reimer *et al*, 1998). However, this hypothesis has not been specifically tested in BB rats.

Despite the ability of certain food components to alter diabetes incidence in the BB rat, with the exception of raw lentils, no single food component added to the casein-based diet has been able to produce the same level of incidence as the standard chow diet (Scott, 1994). It may be one of two things; an undetermined food component not yet characterized in chow that could potentiate the development of diabetes when consumed with diabetogenic proteins. Another possibility is that a component of the semi-purified diet, such as casein is protective.

Mechanisms by which Chow Diet is Diabetogenic and Semi-Purified Diet is Protective

The exact mechanism by which diet exerts its protective or diabetogenic effects in the BB rat is not known. The protective effect of a casein-based diet could be a result of a reduced

exposure to diabetogenic agents in chow at an age where developmental processes in susceptible animals may differ from those of normal rats. Also, diet has been shown to influence indices of immune function that could alter the rate of diabetes. Finally, differences exist in the way the gut and GALT respond to chow feeding in BB rats, and these differences could be attributed to an inability to adapt to a nutritionally complex, cereal-based weaning diet (Reimer *et al*, 1998).

Reduced Exposure to Diabetogenic Agents in Chow

Feeding the casein-based-semi-purified diet may be associated with reduced exposure to diabetogenic agents found in chow, thus conferring protection (Hoorfar *et al*, 1992) (Scott *et al*, 1997). The semi-purified diet does not appear to have a diabetes-suppressing effect, as mixing the chow diet with the semi-purified diet increased the incidence of diabetes in a somewhat dose-dependent manner (Scott *et al*, 1997). Studies conducted in human populations suggested that diabetogens introduced at a critical period of time could influence disease expression (Kostraba *et al*, 1992). Diabetogenic effects of cow"s milk protein in BBdp rats showed to be maximal when the pup was exposed between 13-25 days (Atkinson *et al*, 1988). This suggests that a critical period that may exist when the genetically susceptible individual may become immunized to a certain food antigen. However, Scott et al., (1997) found that there was a much larger age window than what was first proposed. It was observed that BBdp rats fed a casein-based diet between weaning (21 days) and puberty (50 days), then switched to a chow diet produced similar rates in diabetes incidence as BBdp rats fed chow throughout (Scott *et al*, 1997). Rats fed defined diets after 30 days of age (fed rat chow previously) had same incidence of diabetes as purely chow fed rats (Issa-Chergui *et al*, 1988) BBdp rats fed semi-purified casein-based diets until 100 days expressed lower rates of diabetes. They concluded that from early puberty (50 days) to late adolescence (100 days) was a potential window for dietary modification of diabetes.

Alteration in Immune Development in BBdp Rats

At birth, neonatal immunity favours a T_h2 response, shifting towards T_h1 as the infant ages (Adkins, 1999). It is possible that a predominantly T_h1 immune response in an infant or young animal could contribute to an altered immune response to dietary antigens. For example, the Th1 cytokine IFN-γ, when administered to pre-weaned rats, increases gut permeability (Sutas *et al*, 1997). Lower IFN-γ mRNA, and a higher level of IL-10 mRNA in the gut when fish oil was added to chow diet, shows that diet can affect GALT in the BB rat (Kleemann *et al*, 1998). As discussed, exposure to diabetes-promoting food at a time where tolerance is more easily

induced resulted in a down regulation of IFN-γ message in neonatal BBdp animals (Scott *et al*, 2002). This suggests that there is a bias towards a T_h1 environment of the gut in BBdp animals during development that can be modulated by dietary exposure and subsequent tolerization (Scott *et al*, 2002).

It has been shown that maternal diet can affect immune parametres in pre-weaned BBdp animals (Field *et al*, 1999a). Semi-purified, casein-based diet-fed BBdp animals had a higher proportion of T-cells than chow fed counter-parts at 15 days of age, suggesting an early effect on the immune system that could potentially affect the incidence of diabetes (Field *et al*, 1999a). Additionally, in pre-weaned BBdp animals (where the dam is fed chow), compared to BBn animals fed chow, resulted in developmental differences in the gut with respect to proglucagon and glucose transporter mRNA (Reimer *et al*, 1997a).

Changes in Immune Activity

Diet is able to modulate immune parametres in the pancreas of BBdp rats (Li *et al*, 1995b;Scott *et al*, 1997). Feeding chow to BBdp rats resulted in an increased expression of MHC class I antigens on pancreatic β-cells compared to the semi-purified diet (Li *et al*, 1995b). MHC class I expression is usually low or absent on β-cells (Li *et al*, 1995b). It presents antigen to CD8+ cytotoxic T-cells (ref), therefore feeding chow diet may increase the antigenicity of β-cells in the BBdp rat. Adminstration of silica, which is cytotoxic to macrophages, prevents the infiltration of this cell type into the pancreas (Li *et al*, 1995b). However, hyperexpression of MHC class I was still observed in chow fed rats after silica administration (Li *et al*, 1995b). It is possible, that chow, which is higher in carbohydrate and lower in fat, increases insulin secretion, and as a result, may alter the antigenicity of the β-cell, making it more susceptible to immune attack (Scott *et al*, 1997). In agreement with MHC antigen hyper expression, it was shown that IFN-γ mRNA was upregulated in 70-day old BBdp rats fed a chow diet compared to casein (Scott *et al*, 1997). TGF-β was higher in BBdp animals fed the semi-purified diet(Scott *et al*, 1997). These findings suggest an anti-inflammatory type response after feeding a semi-purified diet, or a pro-inflammatory response to chow (Scott *et al*, 1997).

It has been well-documented that diet is able to modulate immune function in the periphery. Immune changes observed in the spleen upon feeding a semi-purified diet compared to a chow diet included a decrease in NK cell activity (6 weeks of age and 4 weeks of age)(Field,

1995), (Field *et al*, 1999a) respectively. Furthermore, a decrease in the proportion of macrophages in the spleen was observed. A decrease in aspartate production and increase in glucose oxidation to CO_2 after mitogen stimulation occurs upon chow feeding (Field, 1995). The above findings point to an alteration in immune function that could confer protection when BBdp animals are fed the semi-purified diet.

Closer to the gut, there have been reported effects of feeding a semi-purified diet (Reimer *et al*, 1998) instead of chow, on cells from the MLN. An increase in ConA stimulated IFN-γ and IL-10 production was observed in the mesenteric lymph nodes in BBdp animals fed the semi-purified diet (Olivares *et al*, 1999). Additionally, feeding a semi-purified diet was reported to alter T-cell phenotypes (CD5+ T-cells), increasing their proportions to levels closer to BBn values, with a concomitant decrease in B-cell and macrophage proportions (Field, 1995). A semi-purified diet is also associated with a decreased lactate and pyruvate production by cells of the MLN, suggesting a lower in vivo activation state (Field, 1995). Additionally, (Scott *et al*, 2000) reported that feeding a semi-purified diet (hydrolysed-casein based) resulted in a lower total energy metabolism in the MLN cells, compared to chow feeding.

Studies examining the effects of the two diets within GALT are limited.. This is surprising since the gastrointestinal tract, as discussed, is the first contact of the immune system with dietary antigens. At the level of the gut, feeding the semi-purified diet resulted in a lower unstimulated incorporation of ^{3}H-thymidine in BBdp rats compared to chow feeding from Peyer's Patch cells (Olivares *et al*, 1999). It was also found that feeding chow diet to BBdp animals yielded higher numbers of IgA-bearing cells in the lamina propria of the jejunum and ileum compared to feeding semi-purified diet (Butler S, 1999). The higher number of IgA+ cells in BBdp animals fed chow suggests that there is an increased exposure to antigen, leading to a greater lymphocyte migration to the lamina propria (Butler S, 1999). The mechanism for this increase is not known, however, it is possible that there is either greater antigen uptake by cells in the Peyer's Patches, as an increased intestinal permeability to macromolecules, was demonstrated by Meddings et al., (1999).

Inability to Adapt to a Complex Weaning Diet by BBdp Rats

At weaning, the gut must adapt to increased antigenic stimuli and changes in nutrient composition (Strobel, 2002). In normal BBn animals, weaning to chow diet as opposed to the

semi-purified diet results in a greater villus height and crypt depth in the jejunum and ileum (Butler S, 1999). Additionally, a greater small intestinal length and weight is associated with chow feeding in healthy animals (Reimer *et al*, 1998). However, feeding chow to BBdp animals did not result in a lengthening and deepening of villus height and crypt depth, respectively (Butler S, 1999) nor did an increase in gut length or weight occur in response to chow (Reimer *et al*, 1998).

The effect of diet on glucose transporters has been shown to be differential between BBn and BBdp animals (Reimer *et al*, 1998). Feeding chow compared to a semi-purified diet to BBn animals resulted in an increase in intestinal glucose transporter mRNA, however, BBdp animals did not respond to chow diet by an increase in transporter mRNA. Proglucagon mRNA is also affected by diet in BBn animals, but not BBdp animals (Reimer *et al*, 1998). When adjusted for total colonic mRNA, chow fed BBn rats had a lower levels of proglucagon mRNA than did casein fed rats (Reimer *et al*, 1998). This suggests that intestinal gene transcripts in BBn animals are influenced by diet, with a possible defect present in BBdp animals to adapt to changing nutrient composition in the diet (Reimer *et al*, 1998).

Feeding chow compared to casein to BBdp and BBn animals also results in a different response by the immune system and GALT. Feeding chow to BBn rats significantly increased PMA & Iono- stimulated incorporation of ^{3}H-thymidine compared to feeding the semi-purified diets, however, did not affect incorporation in BBdp rats (Field *et al*, 1999a). Similar patterns were observed in IL-2 production (Field *et al*, 1999a). As mentioned earlier, feeding chow to BBdp animals results in a significantly higher presence of IgA+ cells in the lamina propria, however, this response was not observed in BBn animals (Butler S, 1999). In fact, IgA+ cell numbers were similar in BBdp and BBn animals fed the semi-purified diet (Butler S, 1999). IL-2 receptor expression was also determined in the lamina propria, however, no observable differences were detected between BBn and BBdp animals (Butler S, 1999). It has been hypothesized that the different gut responses to diet between BBn and BBdp animals are due to the different compositions of complex carbohydrates between the two diets, more specifically, the fibre compositions (Reimer *et al*, 1998). Chow diet contains fibres that are fermented in the large intestine by resident microflora, termed "fermentable fibres", while the semi-purified diet contains only non-nutritive cellulose. In healthy animals, fermentable fibres produce a number of changes in the gut, which will be discussed in the next section. It is possible that BBdp animals

may lack the appropriate mechanisms to adapt to dietary challenges provided by chow diet (Reimer *et al*, 1998).

The Importance of Dietary Fibre

Dietary fibre is an important component in maintaining barrier function in the gut (Frankel *et al*, 1995;Reimer *et al*, 1997b). Diets lacking dietary fibre, such as low-residue enteral feedings or total parenteral nutrition (TPN) provide valuable insight to the necessity of this nutrient. TPN and low-residue enteral diets often have detrimental effects on gut maintenance of both immune protection and nutrient absorption (Deitch *et al*, 1993). TPN is associated with gut atrophy, and a decrease in mucosal integrity which could result in increased intestinal permeability to macromolecules and bacterial translocation (Omura *et al*, 2000;Xu *et al*, 1998). Oat fiber significantly decreased bacterial translocation when administered to rats receiving TPN or elemental diet (Frankel *et al*, 1995). Enteral feeding was reported to significantly decreased bacterial translocation compared to parenteral feeding (Frankel *et al*, 1995).

TPN has been associated with reduced GALT mass, depleted Peyer's Patches, lamina propria and intraepithelial lymphocytes, as well as decreasing the CD4+/CD8+ ratio in the lamina propria (Li *et al*, 1995a). A simultaneous decrease in intestinal IgA levels was also observed (Li *et al*, 1995a;Wu *et al*, 1999;Xu *et al*, 1998). Feeding enteral diet prevented GALT atrophy (Li *et al*, 1995a) and is associated with decreased bacterial translocation from the intestine (Xu *et al*, 1998) (Omura *et al*, 2000).

Fermentable Fibres

Fermentable fibres (FF), are usually complex carbohydrates that are not broken down in the small intestine by digestive enzymes and reach the large intestine intact, where they are subject to microbial degradation (Bouhnik *et al*., 1999) Inulin and related compounds such as fructo-oligosaccharides have been classified as prebiotics, which are non-digestive food ingredients that target the growth and activity of certain bacteria, perceived as beneficial to the host, in the colon (Walker, 2000). Many FF are subject to bacterial degradation, but may not selectively target beneficial bacteria, such as lactic acid bacteria (LAB), so they may not be considered prebiotics (Bouhnik *et al*, 1999). Beneficial anaerobic bacteria in the colon produce a number of by-products when they metabolize FF, such as hydrogen, methane, carbon dioxide, and short-chained fatty acids (SCFA) (Bengmark, 1998;Settle, 1988). The SCFA's acetate, propionate and butyrate account for approximately 83% of all SCFA's. They are produced in a

constant molecular ratio of 60:25:15 respectively and are quickly absorbed in the large intestine (Settle, 1988).

Feeding fermentable fibre has been shown to increase villus height and crypt depth in the gut in healthy dogs, and rats (Massimino *et al*, 1998;Reimer *et al*, 1997b). Compared to cellulose, feeding a fermentable fibre from rhubarb was shown to decrease jejunal permeability to macromolecules ((Reimer *et al*, 1997b). Additionally, an increase in ileal proglucagon mRNA and serum GLP-1 was observed in these animals after fibre feeding in both rats and dogs (Massimino *et al*, 1998;Reimer *et al*, 1997b;Reimer & McBurney, 1996). Proglucagon mRNA results in the expression of glucagon-like-peptide–1 (GLP-1) and GLP-2 (Reimer *et al*, 1998) Proglucagon is produced my the L-cells in the distal ileum and colon (Drucker, 2001). GLP-2 is enterotrophic (Jasleen et al., 2002) and may partially explain the growth of the intestine in response to dietary fiber (Reimer *et al*, 1997a). GLP-1 is an insulin secretagogue and may be of importance in the treatment of type 2 diabetes (Drucker, 2001).

Feeding fermentable fibres may result in a shift in the resident microflora. Those fibres which promote the growth and colonization of LAB are known as prebiotic fibres (Walker, 2000). Feeding prebiotic fibres, such as inulin or oligofructose, shifts the colonic microflora to a composition higher in LAB in both rats and humans (Bouhnik *et al*, 1996;Buddington *et al*, 2002;Le Blay *et al*, 1999). LAB are non-pathogenic, gram-positive bacteria that produce mainly lactic acid (Vanderhoof & Young, 1998). Other metabolites produced by LAB include SCFA, H_2, CO_2. SCFAs have demonstrated many bioactive effects *in vivo*. The physiological effects of short SCFA's include lower gastrointestinal pH (Bengmark, 1998), a fuel source for enterocytes, and increased epithelial cell proliferation (Sakata & von Engelhardt, 1983).

Dietary Fiber and Immune Function

There have been few studies that have investigated the effects of feeding fibres fermented in the large intestine on immune function, particularly in the gut (Schley & Field, 2002). One study found that fermentable fibre feeding significantly altered the structure and function of GALT (Field *et al*, 1999b). Switching 16 adult mongrel dogs from a low fermentable fibre to a high fermentable fibre diet (containing fibres from beet pulp, oligofructose powder and gum arabic) resulted in an increased mitogen response from MLN and IEL's, an increase in CD8+ T-cells in the IEL, LP and PP populations, and an increase in CD4+ T-cells in the MLN and blood(Field *et al*, 1999b). In agreement with these findings, the increase in CD8+ T-cells in the

cecum has been reported by others in rats fed sugar beet fibre (Ishizuka & Tanaka, 2002;Nagai *et al*, 2000), and the increase in CD4+ T-cells from the MLN in rats fed pectin (Lim *et al*, 1997).

Other studies have reported that feeding a number of different fibres fermented in the colon, some known as prebiotic fibres and some that are not established prebiotic fibres may alter parametres or GALT (Schley & Field, 2002). Cytokine production by GALT cells has been shown to be altered by feeding different fibres (Lim *et al*, 1997;Yun *et al*, 1998) For example, there was a higher IFN-γ production by MLN cells in rats and mice, fed pectin (Lim *et al*, 1997), and β-glucan, respectively(Yun *et al*, 1997) (Yun *et al*, 1998). Feeding β-glucan had variable effects on IL-4-secreting cells in the MLN (Yun *et al*, 1997;Yun *et al*, 1998). Other effects include an increase in IgA immunoglobulin production in the mucosa (Lim *et al*, 1997) (Yun *et al*, 1998), MLN (Lim *et al*, 1997) (Yamada *et al*, 1999) and periphery (Lim *et al*, 1997;Yamada *et al*, 1999). IgA was also affected by a number of different fibres, including gum arabic, glucomannin, celfur, lactulose and curdlan, by an increase in IgA+ cells in cecal and small intestinal mucosa (Kudoh *et al*, 1998;Kudoh *et al*, 1999). An increase in IgA in cecal and small intestinal mucosa might indicate an increase in antigen exposure in the gut. This could be due to an increase in antigenic content in the colon, such as bacteria in response to an increased substrate availability.

It is evident that fermentable fibres can alter parametres of GALT. The implications of this are not clear, however, some studies have shown that feeding prebiotic fibres can have positive clinical applications. For example, an anti-inflammatory effect after fructo-oligosaccharide administration was observed in experimentally-induced colitis in mice (Cherbut et al., 2003). Additionally, prebiotics might be relevant in the prevention of allergy (Van den & Veereman-Wauters, 2002), and finally, could protect against gastrointestinal infections (Marteau & Boutron-Ruault, 2002). Modulation of intestinal microflora may partially explain the effects of fermentable fibres on GALT, as there have been numerous studies examining the effect of probiotic bacteria on immune function (Schley & Field, 2002).

Feeding lactic acid bacteria (LAB) can affect many parametres of GALT. *Lactobacillus bulgaricus* was demonstrated to reduce bacterial translocation of *E. Coli* in mice (De Simone *et al*, 1992). Intestinal IgA production is increased after probiotic administration in both humans and mice (Perdigon *et al*, 1990;Takahashi *et al*, 1998) (Tejada-Simon *et al*, 1999). In MLN, one study found an increased response to mitogens (Shu *et al*, 2000). In the Peyer's Patches, there was an increase in the number of B-cells, and an increased response to mitogens (De Simone *et*

al, 1987); Shu et al., 2001) after oral administration of LAB in mice. In rats, Peyer's Patch cells had a higher *in vitro* proliferative response to LAB and a greater mitogen-stimulated production of IFN-γ after yogurt-feeding (Aattour *et al*, 2002). In the periphery, T_h1 cytokine production was increased (Aattour *et al*, 2002;Matsuzaki, 1998) with a concomitant decrease in T_h2 cytokines in splenocytes (Matsuzaki et al., 1998). Immunoglobulin production in the periphery was increased by administration of LAB in humans and mice (Tejada-Simon *et al*, 1999;Yasui *et al*, 1989); (Link-Amster *et al*, 1994) (Malin *et al*, 1996), with the exception of IgE, which decreased upon administration in mice (Matsuzaki *et al*, 1998) and chronic yogurt consumption in the elderly (Van de *et al*, 1999).

It is evident that LAB administration can affect immune function in both GALT and the periphery. The mechanisms by which fermentable fibres may change parametres of GALT likely involve the modulation of intestinal microflora (Schley & Field, 2002). Direct contact of LAB and their products, the production of fermentation by-products, SCFA's are two potential mechanisms whereby GALT is affected by fermentable fibre feeding (Schley & Field, 2002).

As previously discussed, feeding fermentable fibres can alter the composition of intestinal microflora (Bouhnik *et al*, 1996;Buddington *et al*, 2002;Le Blay *et al*, 1999). Prebiotics in particular, are selective substrates for LAB, and it is possible that the increase in LAB,their products and the subsequent contact with GALT could result in a stimulation of GALT cells (Schley & Field, 2002). Contact of GALT with bacteria and their components can occur in a number of ways. Bacterial uptake in the Peyer's Patches can occur through endocytosis by M-cells, which endocytose particulate antigen (Hershberg, 2002). Also, dendritic cells, being powerful antigen-presenting cells, have the ability to sample bacteria from the lumen by extending their dendrites between epithelial cells without disrupting epithelial integrity (Rescigno *et al*, 2001). It is also possible that bacterial/epithelial cell interactions could influence the exposure of GALT to luminal bacteria. LAB, and other beneficial bacteria, may out-compete pathogenic bacteria for receptor-sites on enterocytes, such as CD14 (Martin-Villa *et al*, 1997). CD14 is a receptor site on lymphocytes, or APC for components of gram positive cell walls, such as lipoteichoic acid and muramyl dipeptide (Cleveland *et al*, 1996). The enterocyte (intestinal epithelial cell) is an antigen presenting cell and can present antigens basolaterally via MHC class I or II or non-classical antigen-presenting molecules, such as CD1 or Toll-like receptors to cells of GALT (Hershberg, 2002).

In vitro, cell culture studies have reported that co-culturing LAB or cell-products with cells from the immune system can alter immune cell function. Peyer's Patch cells co-cultured with *Bifidus Breve* resulted in an increased proliferation and antibody production (Yasui & Ohwaki, 1991). Furthermore, it has been demonstrated that culturing a macrophage cell-line with bifidobacteria caused an increase in IL-6 and TNF-α production (Marin et al., 1997; (Park *et al*, 1999)and nitric oxide, and H_2O_2 (Park *et al*, 1999). Culture of CD4+ T-cells with Bifidobacteria increased the production of IL-2 and IL-5 (Marin et al., 1997). *In vivo* evidence exists for the effect of bacterial cell fragments, such as cell-wall components on cells of GALT, increasing phagocytic activity of intraperitoneal macrophages and reticuloendothelial phagocytes, producing similar effects as the administration of live bacteria (Perdigon *et al*, 1988). Cytoplasmic constituents have also been shown to affect IgA production by Peyer's Patch cells similarly to live bacteria (Takahashi *et al*, 1998).

IEC can mediate immune responses in the gut through the secretion of various immune effector molecules when exposed to the proper stimuli. HT-29 cells cultured with LAB did not produce IL-8, a potent chemotactic chemokine involved in the inflammatory response, while incubation with cell components from E. Coli induced significant IL-8 secretion (Lammers et al., 2002). Epithelial cell lines can also affect the function immune cells non-specifically, in particular, increasing IL-10 production in monocytes, *in vitro* when exposed to intestinal bacteria, such as lactobacilli (Haller *et al*, 2002). These findings implicate an anti-inflammatory effect of LAB on intestinal epithelial cells. Although limited *in vivo* evidence exists to support this fact, animal studies investigating the use of LAB in inflammatory bowel diseases have found similar effects (Cherbut *et al*, 2003).

A shift in the intestinal microflora composition resulting from ingestion of fermentable fibres could result in a change in nutrient metabolism in the cecum and colon. SCFA, bacterial fibre fermentation by-products, have been shown to exert an immunomodulatory effect. (Pratt *et al*, 1996), demonstrated that supplementing total parenteral nutrition with SCFA after intestinal resection increased natural killer cell activity. In addition, acetate administered intravenously to cancer patients and healthy individuals resulted in increased blood antibody production, natural killer cell activity and a mixed-lymphocyte reaction (Ishizaka *et al*, 1993). It has been suggested thought that the main effects of SCFA are their use a metabolic substrates by epithelial cells, thereby reducing the use of glutamine (Jenkins *et al*, 1999). Glutamine is an important substrate for lymphocytes (Wu *et al*, 1991) and could therefore be made available.

However, a number of *in vitro* studies suggest that SCFA may also have separate immunomodulatory effects, more specifically, anti-inflammatory effects (Bohmig *et al*, 1997;Inan *et al*, 2000;Saemann *et al*, 2000). Butyrate suppressed the activation of NF-?B by TNF-a but did not affect activation by IL-1ß (Inan *et al*, 2000). NF-?B is a transcription factor that plays a role in regulating the expression of various cytokines and cell adhesion molecules involved in immune and inflammatory responses, as well as apoptosis (Inan *et al*, 2000). Butyrate has been shown to down-regulate the IFN-γ-induced expression of CD80, and other co-stimulatory/adhesion molecules, such as LFA-3 and ICAM-1 on antigen presenting cells (Bohmig *et al*, 1997). This results in an inhibition of primary T-cell activation (Bohmig *et al*, 1997). Additionally, butyrate incubated with human peripheral blood mononuclear cells resulted in an increased secretion of IL-10 (Saemann *et al*, 2000) Butyrate has been shown to halt cell cycle progession at the G1 phase (Gilbert *et al*, 2000). These studies effectively demonstrate the anti-inflammatory effects of the short-chain fatty acid butyrate *in vitro*.

It is evident that dietary fibre can profoundly affect immune function through various mechanisms, both locally in the GALT and peripherally. Fibre, and the resultant shifts in microflora, can alter parametres of gut morphology, hormones and immunity. In a healthy animal, this could be considered as a normal adaptation. Normally, a diet rich in antigens would stimulate the GALT, resulting in tolergenic signals (Strobel, 2002). In an animal presdisposed to autoimmunity, stimulation of GALT in the absence of these signals could further exacerbate the risk.

Summary

It is clear that the pathogenesis of autoimmune diabetes is multifactorial and dependent on both genetic and environmental stimuli. The BB rat is a useful model for human type 1 diabetes that has furthered our understanding of the effects of environmental factors, including diet, on the autoimmune process of diabetes. Research into the immune etiology suggests that there is a defect in immunoregulation, however, what triggers this is yet unknown. As diet is a potent modulator in the outcome of diabetes in animal models, and weaning diet has been suggested to be a risk factor in the development of diabetes in humans, it is possible that a dietary ingredient may influence the development of diabetes. Research into the mechanisms of the pathogenesis of diabetes has largely overlooked the role of the gastrointestinal tract, despite it being the first locus of antigenic contact with respect to diet. Diet-independent research targeting

the gut has implicated cells of GALT in the pathogenesis of diabetes. In the BB rat, diet-related decreases in the incidence is attributed to decreased activation of the immune system, strongly suggesting that antigens found in chow diet are reaching the periphery. Components of chow diet responsible for the increased incidence if diabetes have been well-studied with the exception of dietary fibre. Dietary fibre has repeatedly been shown to affect parametres of immune function and may therefore be implicated in diabetes in the BB rat.

Chapter 2: Rationale

The BB rat develops autoimmune diabetes that is sensitive to dietary modulation. The standard, cereal-based chow diet results in a higher incidence of diabetes compared to feeding a defined, semi-purified, casein-based diet (Scott *et al*, 1985). The diabetogenicity of various dietary components of chow have been defined in BB rats, however, no single ingredient has produced rates of diabetes comparable to chow (Scott, 1994). It follows that there is some yet undefined ingredient in chow either directly responsible for the observed high incidence, or indirectly responsible in combination with other diabetogenic ingredients.

Most of the macronutrients in chow have been well characterized in terms of diabetogenicity in BB rats. The exception being that of complex carbohydrates, such as fibre. Standard chow diet and the semi-purified casein-based diet differ in their fibre composition (Field, 1995). The semi-purified casein-based diet contains 100% non-nutritive cellulose. Standard chow contains many fibres, some of which are fermented in the large intestine by resident microflora, termed fermentable fibres (McBurney et al., 1989).

The effects of fermentable fibres, in particular prebiotic fibres, on the gut of healthy animals have been well-documented. Fermentable fibre feeding has been shown to increase jejunal villus height, proglucagon mRNA, GLP-1 secretion and intestinal glucose transporters (Massimino *et al*, 1998). Additionally, fermentable fibre feeding significantly alters parametres of immune function in the GALT (Field *et al*, 1999b).

In addition to modifying incidence, feeding a semi-purified diet instead of chow has been shown to alter immune function in BBdp animals, whose phenotype is characterized by a number of immune abnormalities, including T-cell lymphopenia and an altered lymphocyte metabolism, which exist prior to diabetes onset (Field *et al*, 1994). Feeding a semi-purified diet has been shown to improve T-cell profiles (Field *et al*, 1999a;Field, 1995) and lymphocyte metabolism (Field, 1995). Contact of the immune system with food antigens first occurs in the gastrointestinal tract, and to date, there have been relatively few studies that have examined the GALT in BB rats, despite marked effects of diet on immune function in this model.

Previous studies have found that BBdp animals do not have the same response at the level of the gut to chow feeding as BBn animals (Reimer *et al*, 1998). Increased gut length, jejunal villus height and crypt depth (Butler S, 1999), levels of proglucagon mRNA, glucose transporters and total colonic mRNA are associated with chow feeding in BBn animals, but not in

BBdp animals (Reimer *et al*, 1998). Parametres of GALT have also shown to be affected differentially in BBn and BBdp animals with chow feeding. Chow feeding increases the number of IgA-bearing cells in lamina propria of the jejunum and ileum in BBdp animals, while feeding semi-purified diet results in similar IgA-bearing cell-counts as BBn animals, which were not affected by diet (Butler S, 1999).

The above findings suggest that there is an inability to respond to a dietary challenge provided by a nutritionally complex, mixed-cereal diet in the BBdp rat. Chow feeding at weaning requires intestinal adaptation as chow introduces a higher antigenic load, different macronutrient content and lower energy density than dams milk (Strobel, 1996). Intestinal adaptation at weaning requires signals from both the gut and GALT in response to luminal contents. Intestinal effects of feeding chow in BBn rats reflect those of fermentable fibre in other healthy animal models, including the effect on proglucagon mRNA, and jejunal villus height (Massimino *et al*, 1998;Reimer *et al*, 1998) (Butler S, 1999). This suggests that it may be the fermentable fibre portion of the diet that is contributing to the observed maladaptation of the gut of BBdp rats to chow (diabetogenic diet).

It is possible that the BBdp rat at weaning (21 days) either cannot produce or respond to the appropriate gut- or GALT- derived signals that are responsible for the intestinal adaptation to diet observed in healthy animals. It is also possible that differences in the way the gut and GALT develops is intrinsically abnormal in the BBdp rat and feeding the chow diet in this case could contribute to a higher incidence.

To address these possibilities, the specific objective of this study was to examine the effects of adding the fermentable fibre, inulin, to a low incidence-producing semi-purified, casein-based diet (FF diet), on the immune system and parametres of GALT in both BBdp and BBn animals. To study the effects of immune system and GALT development in the BB rat, the same parameters were examined at both 21 and 35 days of age, in both BBn and BBdp animals fed a semi-purified, casein-based diet without added inulin (Control diet).

It was hypothesized that:

Hypothesis 1: Feeding the FF diet to BBdp and BBn rats will result in altered peripheral immune and GALT functions compared to feeding the control diet. Specifically, the response of the immune system and GALT to the FF diet will differ between BBn and BBdp animals. The

response of the peripheral immune system and GALT from BBn and BBdp animals fed the control diet will be similar, but not necessarily identical.

Hypothesis 2: The effects on peripheral immune and GALT function from BBdp and BBn animals observed with chow feeding are in part due to the fermentable fibre content of the diet.

Hypothesis 3: Immune development from 21 to 35 days will be different between BBn and BBdp groups fed the control diet.

Chapter 3:Materials and Methods

Animals and Diets

Male and female BB (bio-breeding) normal (BBn) and diabetes-prone (BBdp) rats were obtained from the Department of Agricultural, Food and Nutritional Science Colony, whose breeding stock originated from the Animal Resources Division, Health Protection Branch, Health Canada, Ottawa ON, Canada. Animals were housed in groups of three in plastic shoebox cages in a temperature and humidity-controlled environment, on a 12 hour light/dark cycle. This study was conducted in accordance with the Canadian Council on Animal Care guidelines and was also reviewed and approved by the Animal Policy and Welfare Committee in the Faculty of Agriculture, Forestry and Home Economics (protocol number 2000-24C).

BBn and BBdp rats were fed one of two diets at weaning. Both diets were nutritionally complete, casein-based semi-purified diets that differed only in the fibre composition. The control diet contained non-nutritive cellulose as a fibre source, and the fermentable fiber diet (FF) diet contained both cellulose and a fermentable fibre, inulin (Raftilose Synergy, *Quadra chemicals* LTD, Oreye, Belgium). Both diets were made in our laboratory.

The Nutrient Content of the Diets are shown in table 1.

Table 1 Diet Composition

Table 1
Macronutrient Composition of Experimental Diets[1]

Ingredient	Control Diet %w/w	Fermentable Fiber Diet %w/w
Carbohydrate		
Corn Starch	37.8	37.8
Fat		
Soybean Tallow	13.6	13.6
Safflower Oil	6.2	6.2
Flaxseed Oil	0.72	0.72
Protein		
Casein	27	27
Fiber		
Cellulose	8	3.2
Inulin	0	4.8

[1] Supplemented (per kg of diet) 10g AIN-Vitamin Mix, 50g AIN-Mineral Mix, 2.5 g L-methionine, 6.25 g inositol, 2.75 g choline bitartrate

Table 2 Vitamin Mix Composition (g/kg) [1]

Nutrient	g/kg
p-Aminobenzoic Acid	10
Biotin	0.04
Vitamin B12 (0.1% trituration in mannitol)	3
Calcium Pantothenate	4
Choline Dihydrogen Citrate	487.8
Folic Acid	0.2
Inositol	10
Menadione	0.5
Niacin	4
Pyridoxine HCl	0.5
Riboflavin	0.8
Thiamin HCl	0.5
Vitamin A (500,000 units/g) Vitamin D2 (50,000/g)	4
Dry Vitamin E Acetate (500 U/g)	20
Dextrose	454.6

Table 3 Mineral Mix Composition[2]

Mineral	g/kg
Calcium Carbonate	21
Calcium Phosphate, dibasic	735
Magnesium Oxide	25
Potassium Phosphate, dibasic	81
Potassium Sulphate	68
Sodium Chloride	30.6
Sodium Phosphate, dibasic	21.4
Cupric Citrate	0.46
Ferric Citrate, USP	5.58
Manganese Citrate	8.35
Potassium Iodide	0.0072
Zinc Citrate	1.33
Citric Acid	2.3

[1]Vitamin mix added to diet as 10g/kg (1%w/w)(*Harlan Teklad*, Madison, WI)
[2]Mineral mix (*Harlan Teklad*, Madison, WI) added 50g/kg

Experimental Design

Dams were fed control diet once pregnant and after birth until the pups were 21 days old. All litters were culled to 9 pups. Pups stayed with the dams until 21 days of age when they were weaned. At this time point (21 days), three pups from every litter were anesthetized by halothane

and killed by cervical dislocation. The remaining 6 animals were randomly assigned to either the control diet or the FF diet (3 animals per litter per diet). Animals had access to food and water *ad libitum* at all times throughout this study. Animals were not fasted overnight prior to surgeries, because intestinal contents were necessary for another part of this study. After 14 days fed experimental diets (35 days of age),the remaining rats were anesthetized by halothane and killed by cervical dislocation and samples collected.

Lymphocyte Isolation

At necropsy, spleen, mesenteric lymph nodes (MLN) and guts were removed under sterile conditions and immune cells were isolated as described previously (Field *et al*, 1999a). Spleen and MLN were kept on ice in Krebs-Ringer-Hepes (KRH) buffer containing 0.5% bovine serum albumin (BSA) (*Sigma Chemical Co*., St. Louis, MO), pH 7.4, while the gut was placed in Hank's Balanced Salt Solution (HBSS) (Ca^{2+}, Mg^{2+} free) with added dithiothreitol (DTT)(0.62 g/L) and EDTA (0.58 g/L) to minimize contamination of samples with mucus (Lyscom & Brueton, 1982).

The gut was flushed with approximately 20 ml of sterile HBSS with DTT and Peyer's Patches were located visually and removed. The Peyer's Patches were subsequently placed in HBSS with 0.5% BSA and DTT (0.62 g/L).

The spleen, MLN and Peyer's Patches were pressed through a sterile nylon mesh (100μm) and isolated cells were then centrifuged for 10 minutes at 1500 rpm (228xg) (Beckman J2-HC, *Beckman Instruments*, Palo Alto, CA, USA). Splenocytes were incubated for 3 minutes on ice in a solution containing 155mmol NH_4Cl, 0.1 mmol/ disodium EDTA, and 10mmol $KHCO_3$ to lyse red blood cells. Spleen and MLN lymphocytes were then washed with KRH, pelleted and reconstituted in complete culture media (CCM) (RPMI 1640 with antibiotic and antimycotic, 5% FCS (*GIBCO*, Burnlington, ON, Canada), 25 mmol/L HEPES and 2.5 mmol/L 2-mercaptoethanol, pH 7.4. Viability was assessed using trypan blue and was estimated to be > 95% (*Sigma Chemical Co*., St. Louis, MO).

Mononuclear cell phenotyping

Phenotyping of lymphocyte subsets from the spleen, MLN and Peyer's Patches was performed by immunofluorescence. Mouse monoclonal antibodies were specific for rat lymphocyte determinants as mouse anti-rat IgG. Table 4 describes the specificity of the antibodies used (Field, 1995).

Table 4 Monoclonal Antibody Specificity

Antibody (clone)	Determinant	Cell Subsets Identified
OX19	CD5	Thymocytes
		T-lymphocytes
W3/13	Thymocyte glycoprotein	Thymocytes
		Stem Cells
		Plasma Cells
		T-Lymphocytes
W325	CD4	T-helper lymphocytes
OX8	CD8	T-suppressor/cytotoxic Lymphocytes
		Natural Killer Cells
OX12	Kappa chain of surface Ig	B lymphocytes
OX42	C3bi complement receptor	Macrophages/Monocytes
		Granulocytes
3.2.3		Natural Killer Cells

In each v-bottom well, on a 96-well-plate, 2.5-5 x 10^5 cells from Spleen, MLN and Peyer's Patches were incubated for 30 minutes at 4°C with a monoclonal mouse anti-rat IgG antibody (Ox8, w325, Ox42, w313, Ox12, 3.2.3, Ox19 kindly provided by Dr. Rabinovitch, University of Alberta, Edmonton, AB, Canada, CD3 FITC-conjugated mouse anti-rat was purchased from *PharMingen*, Mississauga, ON, Canada). Cells were then washed 3 times with PBS with 4% FCS. A secondary fluorescent-labeled antibody, fluorescein isothiocyanate-conjugated goat anti-mouse IgG (FITC) (*Cedarlane,* Hornby, Ont,Canada) was applied to the cells and incubated in the dark for 30 minutes at 4°C . For double-labeled samples, a second fluorescent label, anti-mouse phycoerythrocytin (PE) (*Cedarlane,* Hornby, ON, Canada) was applied after a second antibody was incubated. All antibodies were made up in PBS with 4% FCS. In case of background fluorescence due to non-specific binding, two aliquots of each sample were designated to receive FITC or PE without a primary antibody. To reduce non-specific binding of the fluorescent label, heat-inactivated BBn rat serum was added to the FITC and PE in PBS in substitution of FCS. After incubation with designated antibodies, 1 % paraformaldehyde in PBS was used to fix the cells (*Anachemia Science*, Montreal, PQ, Canada).

Relative fluorescence intensity for each antibody was determined using a FACScan (*Becton Dickinson*, Sunnyvale CA, USA). Fluorescent-labeled cells were determined as a percentage of total gated cells, corrected for background fluorescence. Data analysis was performed using Cell-Quest Software (*Becton Dickinson Immunocytometry Systems*, Sunnyvale CA, USA). Below is a table outlining antibody combinations in each tissue (Table 5)

Table 5 Antibody Combinations used for Spleen, MLN and Peyer's Patches

Tissue	Single-labeled Antibodies	Double-Labeled Antibodies
MLN lymphocytes	Ox8, w325, Ox42, Ox12	Ox19/w313
Spleen Lymphocytes	Ox8, w325, Ox42, Ox12, 3.2.3	Ox19/w313
Peyer's Patches Cells	Ox8, w325, Ox12	Ox19/w313

Mitogen Stimulation

Mitogenic response of lymphocytes was estimated by stimulating spleen, and MLN lymphocytes from each diet group with Concanavalin A (ConA) (5μg/ml) (ICN, Montreal, PQ, Canada), Phorbol Myristate Acetate (PMA) (20ng/ml) (*ICN*, Montreal, PQ, Canada) and Ionomycin (0.5nmol/ml) (*Sigma Chemical Co.*, St. Louis, MO), and two infant formula proteins, a hydrolysed whey (NZMP) (1000 μg/ml) protein and a soy protein (7410) (800μg/ml). Both proteins were obtained from (*Wyeth-Ayerst Research*, Radnor, PA, U.S.A). The rate of 3H-thymidine uptake was also measured in unstimulated cells. The cells were counted and diluted to $1.25x10^6$ cells/ml in CCM. The assay was performed in triplicate on 96-well culture plates (200μl /well) and incubated for 68 hours at 37°C and 5% CO_2. Eighteen hours prior to harvesting, cells were pulsed with 37.5 kBq (1 μCi) of ^{3}H-Thymidine (*Amersham Canada*, Oakville, ON, Canada). Cells were harvested using a Packard Filter Mate harvester, and counted on a TopCount NXT plate reader using Microscint Scintillation fluid (*Canberra Packard Canada*, Mississauga, Ont, Canada). Stimulation indices were calculated for each sample as indicated by:

Amount of ^{3}H-thymidine incorporation by stimulated cells / amount of incorporation by unstimulated cells

Cytokine Production

As described above, 1-2 ml of cell suspensions of 1.0 x 10^6 cells/ml were either unstimulated or stimulated with the following mitogens: ConA, PMA & Ionomycin, or NZMP. One-hundred µL of mitogen was added per ml of cell suspension at the same dilutions as the proliferation assay. All samples were incubated at 37°C 5% CO_2 for 72 hours. At 72 hours, cell cultures were spun at 250xg for 5 minutes to collect supernatants. Supernatants were frozen and stored at -70°C until cytokine profiles were determined. Cytokines measured from spleen, and MLN are outlined in Table 4.

Table 6 Cytokines Measured from MLN and Spleen stimulated Lymphocytes

Tissue	Mitogen	Cytokines Measured
Spleen Lymphocytes	Unstimulated	All
	PMA & Ionomycin	IL-2, IFN-γ, IL-10, IL-4
	Concanavalin A	IL-2, IL-10, TGF-β
MLN Lymphocytes	NZMP	IL-10, IL-4, IFN-γ, TNF-α
	Unstimulated	All
	Concanavalin A	IFN-γ, TNF-α, TGF-β
	NZMP	IFN-γ, TNF-α, IL-10, IL-4

Determination of cytokine production was performed using commercial ELISA kits (*BD Biocsciences*, Mississauga, ON, Canada). Flat bottom microtiter plates (Immulon 2 HB *VWR*, Edmonton, AB, Canada) were coated with a capture antibody (purified anti-rat) and incubated overnight at 4°C. Plates were washed and blocked for 1 hour at room temperature with a PBS + 10% FCS buffer to reduce non-specific binding. Recombinant standards and culture supernatants were added (100 µl/well) in duplicate to rows on the plate. Plates were incubated for two hours at room temperature, washed and incubated with biotinylated mouse anti-rat antibody and horseradish peroxidase avidin D for an hour. ABTS Substrate was added for 30 minutes and incubated in the dark at room temperature. An H_2SO_4 stop solution was then added to stop colour development and absorbance was read at 405 nm in a microplate reader (SpectraMax 190, *Molecular Devices,* Sunnyvale CA, USA). The concentration of cytokines for each sample was quantified by comparison to a standard curve from recombinant standards and the mean co-efficient of variance was less than 15 %.

Immunohistochemistry

At necropsy, guts were removed under sterile conditions from the ligament of treitz to the ileocecal valve. Gut lengths were rapidly recorded. For histological evaluation, guts were flushed with PBS (pH 7.4) at 4°C and cut into three equal sections; duodenum, jejunum, and ileum. A 3 cm section was removed from the proximal end of each segment and immediately placed in histological cassettes (*Fisher*, Pittsburgh, Pennsylvania, USA) and immersed in a fixing solution for 48 hours. The fixed segments were then rinsed in PBS pH 7.4 for 24 hours then transferred to 10% w/v phosphate buffered formalin (*Fisher*, Edmonton, AB Canada) for storage for immunohistochemistry. The remaining sections were placed in plastic scintillation vials, and snap frozen in liquid nitrogen and stored at -70°C for another part of this study.

Tissue Preparation

Jejunal tissue samples stored in the 10% w/v phosphate buffered formalin were washed overnight under running tap water. After washing, tissues were dehydrated and cleared through graded ethanol dilutions and xylene, respectively, as outlined in Table 5.

Table 7 Dehydrating and Clearing of Tissues

Sequence	Solution	Time
1	50% (v/v) Ethanol	1 hour
2	70% (v/v) Ethanol	1 hour
3	95% (v/v) Ethanol	1 hour
4	100% (v/v) Ethanol	30 minutes
5	100% (v/v) Ethanol	30 minutes
6	Xylene	30 minutes
7	Xylene	30 minutes

After dehydrating and clearing, tissues were immersed in melted paraffin (<60°C) for a period of 30 minutes to 2 hours to ensure adherence of paraffin to the tissue. The tissue was subsequently sliced, arranged in preheated embedding molds (*Simport*, Saint-Mathieu-de-Beloeil, Quebec, Canada) and embedded in paraffin (*Fisher*, Edmonton, AB, Canada). Embedded samples were rapidly cooled on ice and removed from the embedding mold. Samples were cut into 4-6 μm sections, then placed on glass slides (Histobond,*Fisher*, Edmonton, AB, Canada).

Immunostaining

Tissues were deparaffinized and rehydrated through xylenes and graded ethanols respectively. Table 8 outlines the staining process.

Table 8 Staining Procedure for Immunohistochemistry

Order	Step	Solution	Time
1	Clearing	Xylene	5 minutes
2	Clearing	Xylene	5 minutes
3	Rehydration	100% (v/v)Ethanol	2 minutes
4	Rehydration	100% (v/v) Ethanol	2 minutes
5	Rehydration	95% (v/v) Ethanol	2 minutes
6	Rehydration	70% (v/v) Ethanol	2 minutes
7	Blocking Endogenous Peroxidase Activity	0.3% (v/v) H2O2 in Methanol	12 minutes
8	Wash	Tap Water	5 minutes
9	Antigen Retrieval	2mmol Citrate Buffer pH 6.0 at 95 C	30 minutes
10	Wash /cool down	PBS pH 7.4	20 minutes
11	Blocking Buffer	20% sera in PBS pH 7.4	15-30 minutes
12	Primary Antibody	Diluted in PBS	Overnight 4 C
13	Secondary Antibody	Diluted in PBS	15-30 minutes
14	Enzyme complex	ABC	40 minutes
15	Substrate Complex	DAB solution and H_2O_2	10 minutes
16	Counterstain	Hematoxylin	1 minute
17	wash	Tap water	5 minutes
18	Dehydrate	70% (v/v) Ethanol	2 minutes
19	Dehydrate	95% (v/v) Ethanol	2 minutes
20	Dehydrate	100% (v/v) Ethanol	2 minutes
21	Dehydrate	100% (v/v) Ethanol	2 minutes
22	Clearing	Xylene	5 minutes
23	Clearing	Xylene	5 minutes
24	Mounting	Per mount	-

Slides were deparaffinized and rehydrated as described above. Between each step, slides were washed with PBS pH 7.4. Endogenous peroxidase activity was blocked using a solution of 0.3% H_2O_2 (*Fisher*, Edmonton, AB, Canada) in methanol for 12 minutes. Following this step, samples were subjected to an antigen retrieval procedure, where slides were placed in a 2 Mmol citrate buffer (pH 6) for 30 minutes at 95°C.

Non-specific binding was blocked using either 20% goat serum (Gibco, Burlington, ON, Canada) or 20% FCS, depending on the secondary antibody used. Primary antibodies were applied to the slides and incubated overnight at 4°C in a humidified chamber. To measure IgA in the lamina propria, mouse anti-rat monoclonal antibodies to IgA (*Sigma*, St. Louis, MO, USA)

were used at a dilution of 1:200. The secondary antibody for IgA slides was a biotinylated goat anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA) and was used at a dilution of 1:500. To measure B-7.1 (CD80) goat anti-rat monoclonal antibodies to CD80 (*Cedarlane*, Hornby, ON, Canada) were used at a dilution of 1:50. Secondary antibody to CD80 was biotinylated bovine anti-goat IgG (*Cedarlane*, Hornby, ON, Canada) used at a dilution of 1:100. Secondary antibodies for all samples were applied for 30 minutes.

Control slides were subjected to the same treatment as the stained slides, without addition of a primary antibody, to determine background staining. No background staining was observed in these controls for any of the groups. All dilutions and wash periods were performed using PBS pH 7.4. Peroxidase reagents avidin-biotin complex (ABC) were included in a kit (Vectastain ABC Elite kit, *Vector Laboratories*, Burlingame, CA, USA) and were applied to the tissue sample for 40 minutes. Peroxidase activity was developed with diaminobenzidine (DAB) and hydrogen peroxide applied for 10 minutes (DAB peroxidase substrate kit, *Vector Laboratories*, Burlingame CA, USA). After DAB application, IgA-stained slides were counterstained with Harris' Hematoxylin (*Sigma Diagnostics*, St. Louis, Missouri, USA), dehydrated through graded ethanols and cleared with 2 washes of xylene, and mounted with Permount (*Fisher*, Edmonton, AB, Canada).

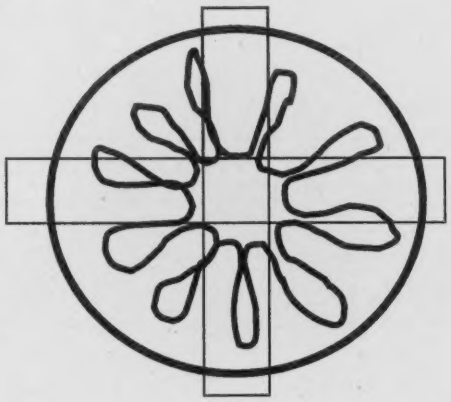
Positive controls for each antibody were done using BBn rat spleen preserved in the same manner as intestinal sections. Isotype controls for IgA were performed using mouse anti-pig CD45RA IgG1 antibodies with sections also processed in the same manner as above. No background staining was observed with isotype controls. Blocking Peptides (*Cedarlane* Hornby, ON, Canada) were applied to the primary antibody to control for background staining CD80 stained slides as these antibodies were polyclonal. No background staining was observed with CD80 plus the blocking peptides.

Immunological Evaluation

All samples were randomized and counted in a blinded manner by the same individual. The microscope used for slide examination was a Leitz Dialux (Wetzlar, Germany) at a magnification of 500X. A computer program was used to visualize the stained tissue samples (MetaMorph, Meta Imaging Series 4.6, *Universal Imaging Corporation*). For IgA-stained samples, four villi per section were selected as shown in figure 1. On each slide, three tissue

sections were examined. Postively-stained cells in the lamina propria region were counted. Sections containing Peyer's Patches were omitted.

Figure 1 Gut Section Demonstrating Villi Selection for Counting IgA+ cells



B-7 (CD80) staining was evaluated by counting the number of positively stained cells at the base of the crypts of Leiberkuhn per 9 crypts. Where staining did not occur in the whole cells, the proportion of positive staining in the lower third of the crypt was determined by MetaMorph. Nine crypts (the same crypts as postively stained cells were counted from) per sample were used to determine mean proportion stained.

Statistical Analysis

All statistical analysis was conducted using the SAS (Statistical Analysis System) statistical software (*version 8.0 SAS Institute Inc.*, Cary, NC, USA). Means are expressed as ±

SEM (n). The level of significance in this study is $p \leq 0.05$. Differences between groups were identified using least square means/pdiff in cases of interactions. Data was first examined to determine if there were any sex effects. Where no sex differences occurred, data was combined and analysed using a two-way ANOVA for effects of age and disease, or diet and disease state. Data likely to be affected by body weight (such as spleen weight and gut weight) were analysed using body weight as a covariate in a 2-way ANOVA.

Chapter 4:Results

Fermentable Fibre: The Effect of Diet and Strain on Immune Parametres in the BB rat

Characteristics of BBn and BBdp Animals at 35 days of Age Fed Different Diets

Body weights differed significantly by sex in both BBdp and BBn animals ($P<0.0001$), therefore, weights were separated and analysed by sex (Table 10). Within a sex, body weight differed significantly only between male BBdp animals, with those fed the FF diet being significantly heavier than those fed the control diet ($P=0.048$). Feeding FF did not significantly change body weights in BBn males or females. Within a sex there was no effect of disease or diet on weight change (Table 10) over a 14 day period. No significant differences were observed in food intake over the 14 day period (BBn control 178±9g, BBn FF 157±14g, BBdp control 166±6g, BBdp FF 145±5g). Spleen weight was affected by strain but not by diet;BBn animals had a greater spleen weight than BBdp animals ($P<0.0001$)(Table 9). The number of lymphocytes isolated from the spleen and MLN was significantly lower in BBdp animals than BBn animals ($P<0.001$), however, BBn and BBdp rats fed the FF diet had significantly fewer splenocytes than their control diet-fed counterparts ($P=0.026$).

As illustrated in Table 9 and Figure 2, small intestinal (gut) length in 35-day-old animals was affected by feeding the FF diet in both BBn and BBdp animals. In BBn animals, feeding the FF diet resulted in a significantly longer gut length (85 ±1 cm) than feeding the control diet (81±1 cm) ($P=0.0031$). Gut length in BBdp and BBn animals fed control diet did not differ significantly.

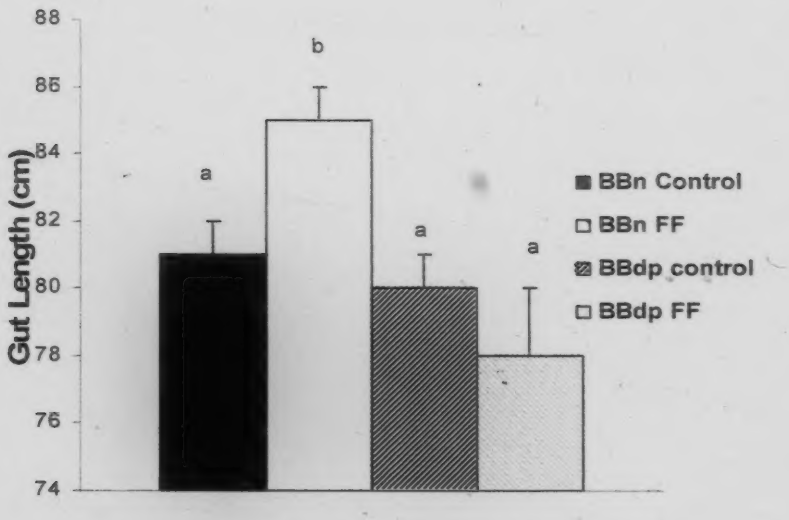


Figure 2 Small intestinal length from 35 –day-old BBn and BBdp rats fed different diets.

BBn control and FF groups (n=26) BBdp control (n=24) BBdp FF (n=25)
Values are presented as mean±SEM. Bars that do not share a common letter are significantly different

Table 9 Characteristics of BBn and BBdp Animalsat 35 Days of Age Fed Different Diets[1,2]

Strain	*BBn*	*BBn*	*BBdp*	*BBdp*	*Significance*			
Diet	Control	FF	Control	FF	Diet	Strain	Interaction (Diet*Strain)	Sex
Gut Length (cm)	81 ± 1 (26)[a]	85 ± 1 (26)[b]	80 ± 1 (24)[a]	78 ± 2 (25)[a]	NS	P<0.0001	P=0.0031	NS
Spleen Weight (mg)	531 ± 18 (24)[a]	537 ± 30 (23)[a]	400 ± 24(22)[b]	397 ± 14(24)[b]	NS	P<0.0001	NS	
# Splenocytes ($x10^6$)	154 ± 12 (25)[a]	129 ± 15 (26)[b]	109 ± 11 (24)[c]	76 ± 8 (24)[d]	P=0.026	P<0.0002	NS	NS
# Splenocytes ($x10^6$)/mg Spleen	263 ± 18 (22)	218 ± 20 (18)	274 ± 41 (17)	193 ± 21 (19)	NS	NS	NS	NS
# MLN cells ($x10^6$)	23 ± 2(25)[a]	21 ± 2 (26)[a]	7 ± 1(25)[b]	8 ± 2(23)[b]	NS	P<0.001	NS	
# Peyer's Patch Cells ($x10^6$)	6.3 ± 0.4 (12)	5.8 ± 0.6 (11)	5.4 ± 0.7 (13)	5.1 ± 0.8 (10)	NS	NS	NS	

Table 10 Body Weight Change of BBn and BBdp Animals Fed Different diets Seperated by Sex[1,2,3]

Strain	*BBn*		*BBdp*		*Significance*		
Diet	FF	Control	FF	Control	Diet	Strain	Sex
Weight Change (g)	61 ± 2 (32)	60 ± 2 (30)	64 ± 2 (38)	62 ± 2 (36)	NS	NS	P<0.0001
Male	66 ± 3 (15)[a]	66 ± 3 (15)[a]	69 ± 2 (17)[a]	64 ± 3 (14)[ab]	NS	NS	
Female	54 ± 3 (17)[b]	55 ± 3 (15)[b]	60 ± 2 (21)[b]	61 ± 2 (21)[b]	NS	NS	

[1] Values presented as mean±SEM (n)
[2] Within a row, values that do not share the same superscript are significantly different ($p<0.05$)
[3] Within a column, values that do not share the same superscript are significantly different ($p<0.05$)

Spleen Phenotypes: The Effect of Diet and Strain on Lymphocyte Subsets in Animals 35 Days of Age

The percentage of T-cells (W3/13+, Ox19+W3/13+), CD4+ (W3/25+) was lower and B-cells (Ox12+) and monocytes/macrophages (Ox42+) was higher in BBdp compared to BBn animals ($P<0.0001$) (Table 11). There were no differences in the proportions of Ox19+ T-cells, CD8+ (Ox8+) T-cells and the CD4/CD8 ratio (W3/25/Ox8) between strains. As illustrated in Table 11, no significant diet differences were observed in spleen lymphocyte phenotypes between BBn and BBdp animals.

Mesenteric Lymph Node Phenotypes: The Effect of Diet and Strain on Lymphocyte Subsets in 35-day-old Animals

All T-cell markers, Ox19+, W313+, Ox8+ and W325+ lymphocyte subsets were significantly higher in BBn animals than in BBdp animals ($P<0.0001$) (Table 12). The percentage of Ox12+, and Ox42+ cells was greater in BBdp animals than in BBn animals ($P<0.0001$). Diet did not affect lymphocyte subsets in 35-day-old BBn and BBdp animals, as illustrated in Table 12, the only exception was the W3/25:Ox8 ratio, which was significantly higher in BBdp, but not BBn, rats fed the control diet ($P=0.045$).

Peyer's Patch Phenotypes: The Effect of Diet and Strain on Lymphocyte Subsets in 35-day-old Animals

BBn animals had a significantly higher percentage of T-cell markers, Ox19+, W3/13+, Ox19+/W3/13+, Ox8+, and W3/25+ lymphocytes than BBdp animals ($P=0.02$) (Table 13). Feeding the FF diet to BBn animals resulted in a significantly higher percentage of Ox8+ lymphocytes in the Peyer's Patches compared to feeding the control diet ($P=0.03$) (Figure 3). BBdp animals had virtually no Ox8+ lymphocytes in the Peyer's Patches regardless of diet ($P<0.0001$). A lower W3/25:Ox8 ratio in BBn fed the FF diet was observed compared to feeding the control diet ($P=0.03$). In BBdp animals, but BBn animals, feeding the FF diet resulted in a significantly greater proportion of Ox12+ (B) cells ($P=0.01$) (Figure 4).

Table 11 Spleen Phenotypes: Effect of Diet and Strain in 35-Day Old Animals [1,2]

Strain	*BBn*		*BBdp*		*Significance*		
Diet	Control	FF	Control	FF	Diet	Strain	Interaction (Diet*Strain)
Ox19+	29 ± 2 (16)	31 ± 3 (13)	22 ± 5 (7)	23 ± 5 (9)	NS	NS	NS
W3/13+	30 ± 3 (11)^{a}	32 ± 3 (13)^{a}	19 ± 3 (7)^{b}	19 ± 2 (10)^{b}	NS	P=0.0002	NS
Ox19+W3/13+	22 ± 3 (13)^{a}	29 ± 2 (10)^{a}	11 ± 2 (7)^{b}	14 ± 3 (10)^{b}	NS	P<0.0001	NS
Ox8+	14 ± 1 (15)^{a}	16 ± 2 (15)^{a}	9 ± 1 (11)^{b}	12 ± 1 (9)^{ab}	NS	P=0.0191	NS
W3/25+	22 ± 1 (14)^{a}	21 ± 2 (15)^{a}	11 ± 2(11)^{b}	13 ± 1 (11)^{b}	NS	P<0.0001	NS
Ox12+	22 ± 2 (13)^{a}	20 ± 1 (14)^{a}	37 ± 3 (11)^{b}	43 ± 3 (10)^{b}	NS	P<0.0001	NS
W3/25:Ox8	1.7 ± 0.1 (13)	1.7 ± 0.3 (15)	1.4 ± 0.2 (10)	1.1 ± 0.1 (9)	NS	NS	NS
Ox42+	8 ± 1 (13)^{a}	7 ± 1(14)^{a}	13 ± 2 (11)^{b}	12 ± 1 (9)^{b}	NS	P=0.0002	NS
323+	9 ± 1 (14)^{ab}	7 ± 1 (14)^{a}	8 ± 2 (7)^{ab}	13 ± 2 (9)^{b}	NS	NS	P=0.022

Table 12 Mesenteric Lymph Node Phenotypes: The Effect of Diet and Strain in 35 Day-Old Animals [1,2]

Strain	*BBn*		*BBdp*		*Significance*		
Diet	Control	FF	Control	FF	Diet	Strain	Interaction (Diet*Strain)
Ox19+	73 ± 2 (13)^{a}	69 ± 2 (12)^{a}	25 ± 1 (14)^{b}	24 ± 2 (18)^{b}	NS	P<0.0001	NS
W/313+	74 ± 3 (14)^{a}	72 ± 3 (11)^{a}	27 ± 2(11)^{b}	26 ± 2(12)^{b}	NS	P<0.0001	NS
Ox19+W/313+	62 ± 3 (15)^{a}	66 ± 1 (11)^{a}	20 ± 1 (14)^{b}	20 ± 1 (12)^{b}	NS	P<0.0001	NS
Ox8+	20 ± 0.6 (17)^{a}	20 ± 2 (14)^{a}	4 ± 1 (12)^{b}	6 ± 1(14)^{b}	NS	P<0.0001	NS
W/325+	56 ± 2 (12)^{a}	54 ± 2 (13)^{a}	20 ± 2 (16)^{b}	23 ± 2(16)^{b}	NS	P<0.0001	NS
Ox12+	15 ± 1 (15)^{a}	15 ± 1(12)^{a}	45 ± 3 (16)^{b}	41 ± 3 (16)^{b}	NS	P<0.0001	NS
W/325:Ox8	3 ± 0.08 (11)^{a}	3 ± 0.4 (12)^{a}	7 ± 1.8 (11)^{b}	4 ± 0.4 (11)^{c}	NS	P=0.004	P=0.045
Ox42+	5 ± 0.6 (15)^{a}	3 ± 0.6 (9)^{a}	9 ± 2 (11)^{b}	9 ± 2 (6)^{b}	NS	P<0.0001	NS

[1] Values presented as proportion (%) of total gated cells; mean ± SEM (n)
[2] Within a row, values that do not share the same superscript are significantly different ($p<0.05$)

Table 13 Peyer's Patch Phenotypes: The Effect of Diet and Strain in 35-day-old Animals [1,2]

Strain	*BBn*		*BBdp*		*Significance*		
Diet	Control	FF	Control	FF	Diet	Strain	Interaction (Diet*Strain)
Ox19+	22 ± 2 (7)[a]	24 ± 1 (12)[a]	10 ± 1 (9)[b]	9 ± 1 (11)[b]	NS	P=0.02	NS
W3/13+	20 ± 2 (6)[a]	23 ± 2 (9)[a]	9 ± 2 (7)[b]	10 ± 2 (8)[b]	NS	P<0.0001	NS
Ox19+/W3/13+	16.2 ± 2.0 (7)[a]	18.1 ± 1.0 (10)[a]	4.6 ± 0.3 (7)[b]	4.3 ± 0.3 (8)[b]	NS	P<0.0001	NS
Ox8+	4 ± 0.9 (10)[a]	10 ± 0.9 (9)[b]	0.3 ± 0.2 (9)[c]	0.8 ± 0.3 (9)[c]	P=0.003	P<0.0001	P=0.011
W3/25+	18 ± 2 (10)[a]	15 ± 1 (10)[a]	7 ± 1 (9)[b]	7 ± 1 (10)[b]	NS	P=0.04	NS
Ox12+	55 ± 2 (9)[a]	54 ± 2 (12)[a]	54 ± 2 (9)[a]	63 ± 2 (10)[b]	P=0.01	NS	NS
W325:Ox8	4 ± 0.8[a]	2 ± 0.4[b]	.	.	P=0.03	NA	NA

[1] Values presented as proportion (%) of total gated cells; mean ± SEM (n)
[2] Within a row, values that do not share the same superscript are significantly different ($p<0.05$) NA=Not applicable

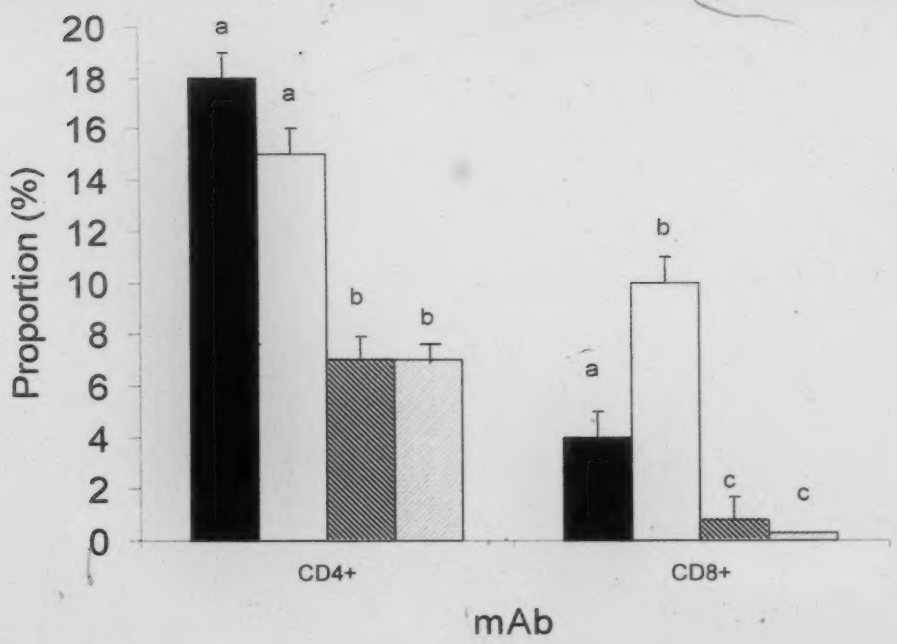


Figure 3 T-cell subsets in the Peyer's Patches of BBn and BBdp rats fed different diets at 35 days of age. BBn control (n=10) BBn FF (n=9) BBdp control (n=9) BBdp FF (n= 9).Values presented as mean ± SEM. Bars within a mAB that do not share the same letter are significantly different (P<0.05).

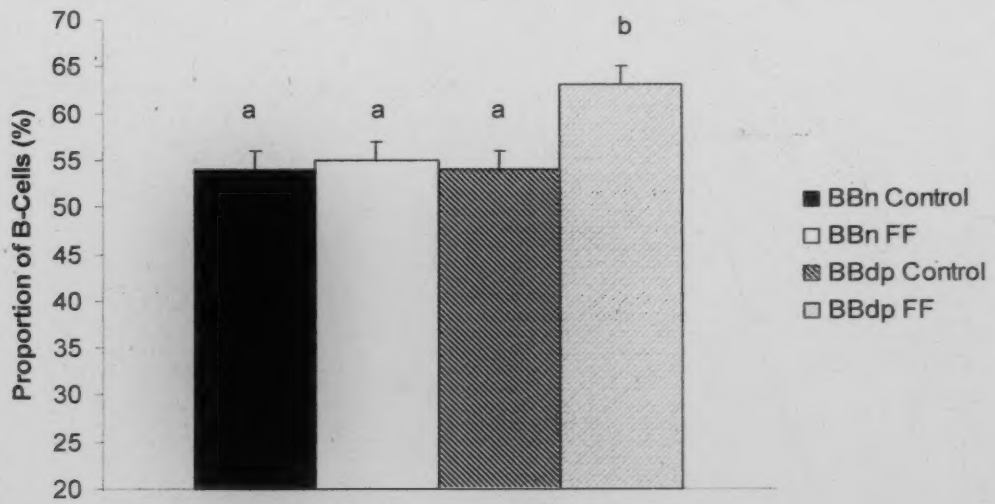


Figure 4 Proportion of B-cells in the Peyer's Patches from BBn and BBdp animals fed different diets at 35 days-of-age. BBn control (n=9) BBn FF (n=12) BBdp control (n=9) BBdpFF (n=10).Values presented as mean±SEM. Bars within a mAB that do not share the same letter are significantly different (P<0.05).

Spleen Lymphocytes: Rate of ^{3}H-thymidine Uptake at 35 days of Age in BBn and BBdp Animals Fed Different Diets

Splenocytes, in the absence of a mitogen, showed no significant difference in the ^{3}H-thymidine uptake between BBn and BBdp groups fed either the control diet or the FF diet (Table 14). In lymphocytes stimulated with ConA or PMA and Ionomycin, there was a significant effect of animal strain ($P<0.0001$), with splenocytes from BBn rats having both a higher ^{3}H-thymidine incorporation and a greater stimulation index (SI) than BBdp splenocytes ($P<0.0001$) (Table 14). There was no significant difference in ^{3}H-thymidine uptake in splenocytes incubated with hydrolysed whey protein (NZMP) or soy protein. In splenocytes from BBn animals stimulated with PMA and Ionomycin, feeding the FF diet resulted in a higher ^{3}H-thymidine uptake ($P=0.038$) and a higher SI ($P=0.001$) compared to feeding the control diet (Table 14, Figure 5). This effect was not observed in splenocytes from BBdp animals. Incubation of splenocytes with hydrolysed whey resulted in a significantly lower SI in BBn animals fed the FF diet compared to the control diet ($P=0.01$). This diet effect was not observed in BBdp animals.

Spleen Lymphocytes: Mitogen Stimulated Cytokine Production

Unstimulated splenocytes did not produce IL-2 (data not shown). When stimulated with both ConA and PMA & Ionomycin, splenocytes from BBn animals produced significantly more IL-2 than splenocytes from BBdp animals ($P<0.0001$) (Table 15, Figure 6). No significant differences due to diet in either strain were observed. There was no significant difference in the production of PMA & Ionomycin-stimulated IFN-γ between strains or diets (Table 15, Figure 7). IL-10 was produced by unstimulated cells, but production did not differ amongst groups. A significant strain effect was present, with BBn animals producing more IL-10 after stimulation with ConA ($P=0.03$) (Figure 8). Feeding FF to BBdp rats resulted in rates of IL-10 production that were not different from BBn rats (Figure 8). When stimulated with ConA, there was a significant diet effect in both BBn and BBdp animals, with FF feeding resulting in an increased IL-10 production by splenocytes, when compared to feeding control diet ($P=0.009$) (Table 15, Figure 8). Significant effects of strain and diet were observed with PMA & Ionomycin stimulated IL-4 production, with BBn animals producing larger amounts of IL-4 than BBdp animals ($P=0.02$), and both strains producing significantly less IL-4 when fed the FF diet ($P=0.02$)(Table 15, Figure 9). Incubating splenocytes with NZMP, resulted in a production of IFN-γ or TNF-α that varied considerably between animals, and did not result in production of IL-

2 (data not shown). However, after incubation with NZMP, IL-10 production, while not different from the unstimulated production, was significantly higher in BBn animals than in BBdp animals ($P<0.0001$) (Table 15).

Table 14 Spleen Lymphocytes: Rate of ^{3}H-thymidine Uptake at 35 days of Age in BBn and BBdp Animals Fed Different Diets [1, 2]

Strain		*BBn*		*BBdp*		*Significance*		
Diet		**Control**	**FF**	**Control**	**FF**	**Diet**	**Strain**	**Interactio (Diet*Stra**
Mitogen								
Unstimulated	***DPM*** [3]	1756 ± 248 (27)	1841 ± 239 (28)	1933 ± 197 (16)	1941 ± 213 (13)	NS	NS	NS
ConA	***DPM***	100072 ± 24220 (8)[a]	87896 ± 15174 (8)[a]	4833 ± 1665 (9)[b]	9571 ± 4410 (10)[b]	NS	P<0.0001	NS
	SI [4]	39 ± 6 (8)[a]	44 ± 3 (8)[a]	4 ± 2 (9)[b]	5 ± 3 (10)[b]	NS	P<0.0001	NS
PMA& Iono	***DPM***	51529 ± 12820 (16)[b]	85261 ± 12383 (13)[b]	9998 ± 467 (16)[c]	13736 ± 2713 (16)[c]	P=0.038	P<0.0001	NS
	SI	19 ± 2 (16)[a]	37 ± 6 (13)[b]	5 ± 1 (16)[c]	7 ± 2 (16)[c]	P=0.001	P<0.0001	P=0.006
Hydrolysed	***DPM***	1083 ± 184 (16)	1011 ± 211 (16)	1722 ± 293 (6)	1577 ± 110 (5)	NS	NS	NS
Whey (NZMP)	***SI***	0.9 ± 0.1 (16)[a]	0.6 ± 0.1 (16)[b]	1.1 ± 0.1 (6)[a]	0.9 ± 0.1 (5)[a]	P=0.01	P=0.02	NS
SOY	***DPM***	575 ± 51 (10)	723 ± 126 (12)	608 ± 267 (4)	982 ± 157 (5)	NS	NS	NS
	SI	0.91 ± 0.06 (10)	0.72 ± 0.07 (12)	0.79 ± 0.12 (4)	0.51 ± 0.03 (5)	NS	NS	NS

[1] Values presented as mean±SEM (n)
[2] Within a row, values that do not share the same superscript are significantly different ($p<0.05$)
[3] DPM (Decays per minute)
[4] SI (stimulation index calculated as DPM stimulated/DPM unstimulated

Table 15 Mitogen Stimulated Cytokine Production By Splenocytes from BBn and BBdp Animals Fed Different Diets [1,2]

	Strain	*BBn*		*BBdp*		*Significance*		
	Diet	Control	FF	Control	FF	Diet	Strain	Interaction (diet*strain)
Mitogen	Cytokine							
Unstimulated	IL-10	336 ± 56(11)	406 ± 66 (12)	291 ± 29 (14)	264 ± 44	NS	NS	NS
ConA	IL-2	2420 ± 324 (8)^{a}	2395 ± 295 (11)^{a}	393 ± 64 (8)^{b}	487 ± 74 (10)^{b}	NS	p<0.0001	NS
	IL-10	652 ± 29 (10)^{a}	904 ± 107 (10)^{b}	471 ± 43(10)^{c}	738 ± 123 (12)^{d}	p=0.009	p=0.03	NS
	TGF-β	128 ± 20 (12)	147 ± 32 (11)	161 ± 23 (10)	128 ± 14 (13)	NS	NS	NS
PMA & Iono	IL-2	4871 ± 191 (6)^{a}	4029 ± 342(6)^{a}	1782 ± 260(9)^{b}	1628 ± 275 (10)^{b}	NS	p<0.0001	NS
	IFN-γ	591 ± 95 (8)	520 ± 187 (8)	890 ± 192 (8)	545 ± 101(12)	NS	NS	NS
	IL-10	690 ± 207 (9)	798 ± 99 (13)	476 ± 51 (9)	419 ± 51 (11)	NS	NS	NS
	IL-4	35 ± 6(10)^{a}	21 ± 4(7)^{b}	21 ± 6(10)^{b}	10 ± 2(9)^{c}	p=0.02	p=0.02	NS
NZMP	IL-10	469 ± 51(17)^{a}	444 ± 61(14)^{a}	224 ± 37(11)^{b}	276 ± 27(15)^{b}	NS	p<0.0001	NS

[1]Values presented as pg/ml mean±SEM (n)

[2] Within a row, values that do not share the same superscript are significantly different ($p<0.05$)

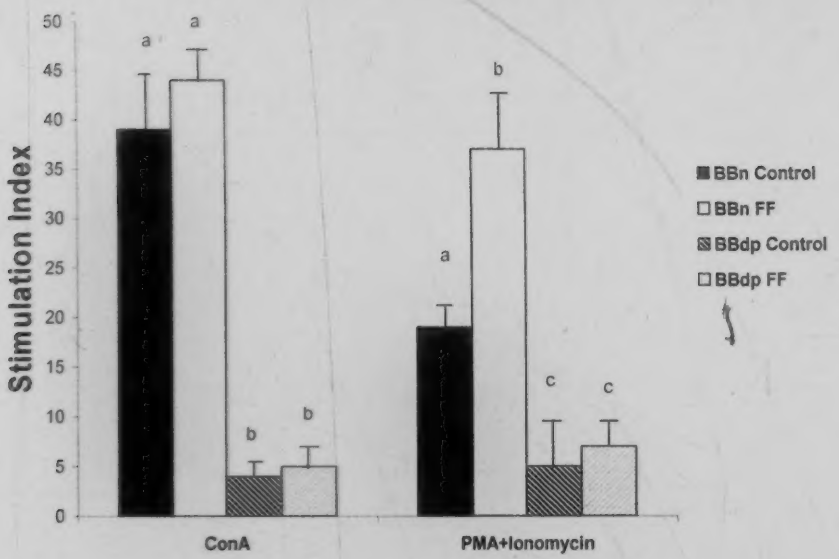


Figure 5 Spleen Lymphocytes: Rate of ^{3}H-thymidine Uptake at 35 days of Age in BBn and BBdp Animals Fed Different Diets. ConA: BBn control (n=8) BBn FF (n=8) BBdp control (n=9) BBdp FF (n=10). PMA & Iono: BBn control (n=16)BBn FF (n=13) BBdp control (n=16) BBdp FF (n=16). Values presented as mean±SEM. Bars that do not share a common letter are significantly different within a mitogen ($p<0.05$).

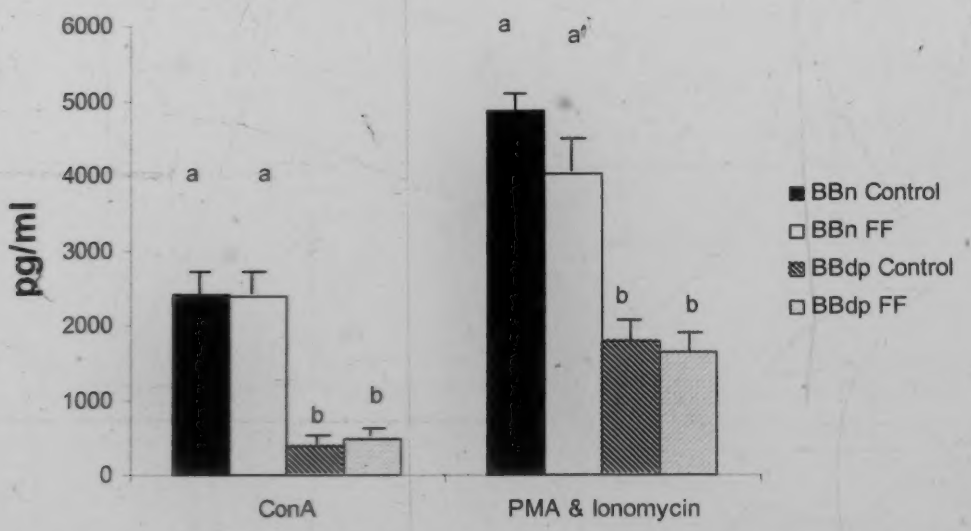


Figure 6 Spleen: Mitogen-Stimulated IL-2 Production: Effect of Diet and Disease

ConA: BBn control (n=8) BBn FF (n=11) BBdp control (n=8) BBdp FF (n=10) PMA & Iono: BBn control (n=6) BBn FF (n=6) BBdp control (n=9) BBdp FF (n=10).Values presented as mean±SEM. Bars that do not share a common letter are significantly different, within a mitogen ($p<0.05$).

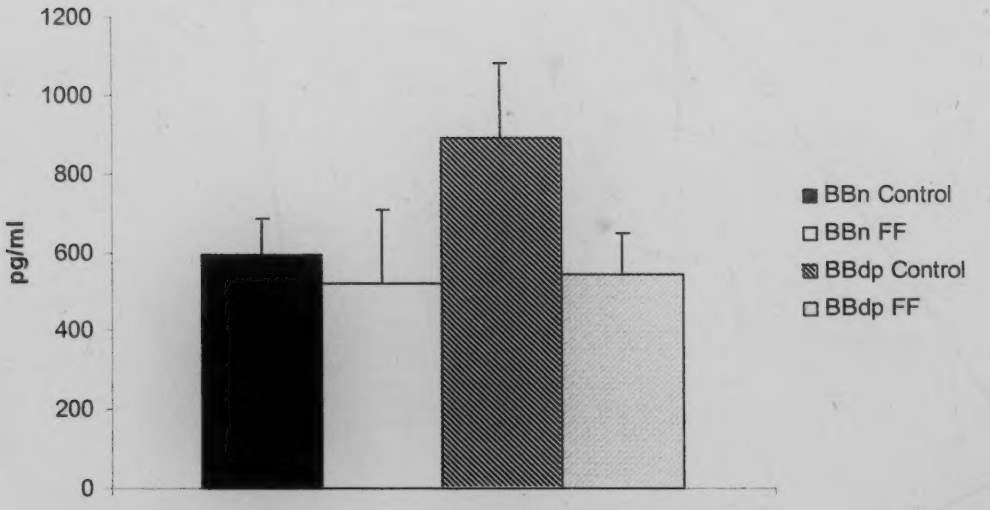


Figure 7 Spleen: PMA & Ionomycin-Stimulated IFN-γ Production ConA: BBn control (n=8) BBn FF (n=8) BBdp control (n=8) BBdp FF (n=12) Values presented as mean±SEM. No significant differences found between groups at $p<0.05$.

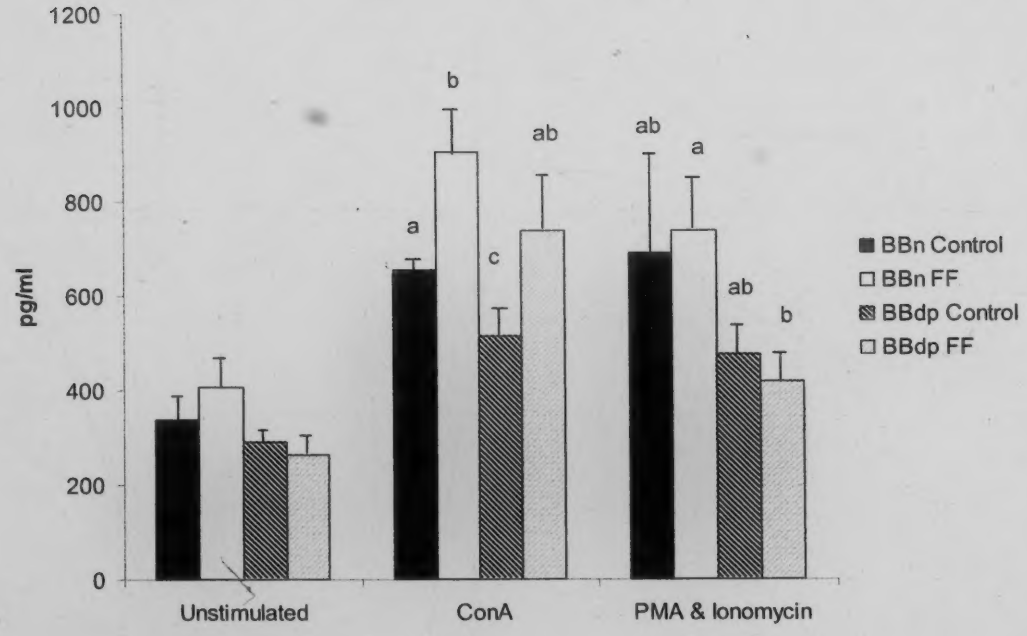


Figure 8 Spleen:Mitogen-Stimulated IL-10 Production

Values presented as mean±SEM. Bars that do not share a common letter are significantly different within a mitogen ($p<0.05$)

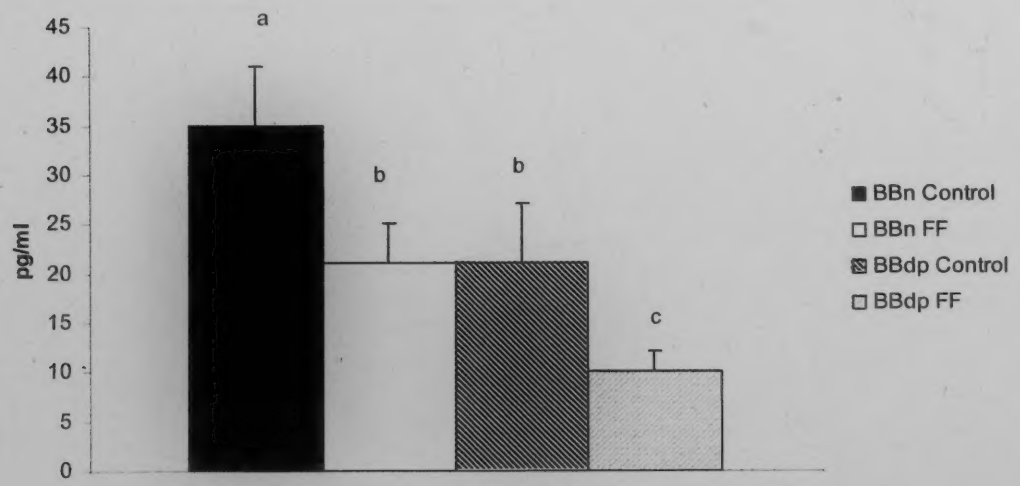


Figure 9 Spleen:PMA & Ionomycin-stimulated IL-4 production. . BBn control (n=10) BBn FF (n=7) BBdp control (n=10) BBdp FF (n=9). Values presented as mean±SEM. Bars that do not share a common letter are significantly different ($p<0.05$).

Mesenteric Lymph Nodes: ^{3}H-thymidine Uptake in 35-Day-Old BBn and BBdp Animals Fed Different Diets

In ConA-stimulated MLN lymphocytes, a significant effect of rat strain was observed ($P<0.0001$) with BBn animals showing greater ^{3}H-thymidine uptake (decays per minute-DPM) than BBdp animals (Table 16). For BBn rats, PMA and Ionomycin stimulation resulted in a significantly higher SI in the group fed the FF diet compared to the control diet ($P=0.038$) but this diet effect was not seen in MLN lymphocytes from BBdp animals fed the FF diet. MLN lymphocytes from both BBn and BBdp animals fed the control diet did not differ in the response to PMA and Ionomycin stimulation (Table 16, Figure 10). There was no effect of diet or strain on the response of MLN lymphocytes to NZMP or soy proteins.

Mesenteric Lymph Nodes: Mitogen-Stimulated Cytokine Production

BBn animals produced significantly more ConA-stimulated IFN-γ than BBdp animals ($P=0.004$) (Table 17,Figure 11). Diet did not affect cytokine production amongst groups with the exception of unstimulated IL-10 production by BBn cells. Feeding the FF diet resulted in a significantly higher IL-10 production than feeding the control diet diet by unstimulated BBn MLN cells ($P=0.007$) (Table 17). Neither ConA-stimulated TNF-α, nor TGF-β production differed significantly amongst groups (Figures 12 and 13, respectively). Cells incubated with NZMP did not differ in their ability to produce IL-10 or TGF-β.

Peyer's Patches: Mitogenic Response

Cells isolated from Peyer's Patches did not proliferate in response to any mitogens (SI=0 for all BBn and BBdp groups). The presence of mucous after 72 hours of incubation for both proliferation and cytokine assays suggests contamination of lymphocyte cultures with goblet cells. Cells from Peyer's Patches did not produce IL-2, IFN-γ, or TNF-α. ConA-stimulated TGF-β was produced at levels higher than in unstimulated cultures (Figure 13), however, no significant differences were observed amongst diet/disease groups. Mean±SEM TGF-β production by each group was; BBn Control 147±28, BBn FF 162±10, BBdp Control 151±44, BBdp FF 226±46 (Figure 13).

Table 16 Mesenteric Lymph Nodes: ^{3}H-thymidine Uptake in 35 Day Old BBn and BBdp Animals

Strain		*BBn*		*BBdp*		*Significance*		
Diet		Control	FF	Control	FF	Diet	Strain	Interaction (diet*disease)
Mitogen								
Unstimulated	*DPM*	558 ± 196 (19)	524 ± 123 (28)	528 ± 92 (20)	750 ± 134 (20)	NS	NS	NS
ConA	*DPM*	136518 ± 23874 (13)	146254 ± 23407(15)	27856 ± 7742 (16)	17735 ± 4514 (15)	NS	NS	NS
	SI	249 ± 36 (13)[a]	307 ± 32 (15)[a]	45 ± 11 (15)[b]	28 ± 6 (15)[b]	NS	P<0.0001	NS
PMA& Iono	*DPM*	55235 ± 15544 (11)	92804 ± 31095 (13)	70142 ± 18043 (12)	73266 ± 10754 (15)	NS	NS	NS
	SI	196 ± 28 (8)[a]	337 ± 72 (10)[b]	155 ± 29 (12)[a]	146 ± 27 (13)[a]	P=0.038	P=0.01	NS
Hydrolysed	*DPM*	784 ± 297 (16)	870 ± 285 (18)	526 ± 212 (6)	874 ± 380 (6)	NS	NS	NS
Casein (NZMP)	*SI*	1.2 ± 0.1 (16)	1.3 ± 0.4 (18)	1.2 ± 2.1 (6)	0.9 ± 0.1 (6)	NS	NS	NS
SOY	*DPM*	299 ± 65 (4)	449 ± 105 (10)	147 ± 24 (2)	682 ± 256 (2)	NS	NS	NS
	SI	1.2 ± 0.1 (4)	1.1 ± 0.2 (10)	0.7 ± 0.6 (2)	0.9 ± 0.7 (2)	NS	NS	NS

[1] Values presented as mean±SEM (n)
[2] Within a row, values that do not share the same superscript are significantly different ($p<0.05$)\
[3] DPM (Decays per minute)
[4] SI (stimulation index calculated as DPM stimulated/DPM unstimulated

Table 17 Mesenteric Lymph Nodes: Mitogen-Stimulated Cytokine Production [1,2]

	Strain	BBn		BBdp		Significance		
	Diet	Control	FF	Control	FF	Diet	Strain	Diet*Strain
Mitogen	Cytokine							
Unstimulated	IL-10	54 ± 36 (9)[a]	261 ± 71 (7)[b]	144 ± 42 (6)[b]	199 ± 49 (9)[b]	NS	NS	P=0.016
ConA	IFN-γ	1782 ± 242 (10)[a]	1620 ± 287(12)[a]	987 ± 299 (9)[b]	790 ± 185(9)[b]	NS	P=0.004	NS
	TNF-α	469 ± 67(14)	436 ± 58(16)	320 ± 34(7)	345 ± 85(9)	NS	NS	NS
	TGF-β	408 ± 113 (8)	629 ± 115(6)	810 ± 168 (8)	836 ± 38 (9)	NS	NS	NS
NZMP	IL-10	151 ± 49 (12)	125 ± 21(14)	115 ± 24(10)	153 ± 33 (11)	NS	NS	NS
	TGF-β	60 ± 11(8)	35 ± 7(9)	55 ± 5(6)	51 ± 4(4)	NS	NS	NS

[1]Values presented as pg/ml mean±SEM (n)
[2] Within a row, values that do not share the same superscript are significantly different ($p<0.05$)

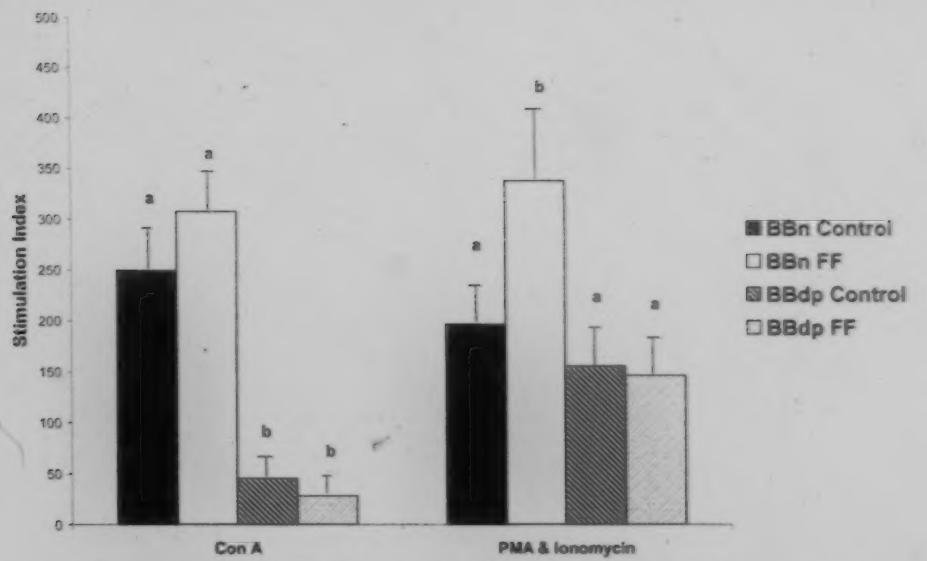


Figure 10 Mesenteric Lymph Nodes: ^{3}H-thymidine Uptake in 35 Day Old BBn and BBdp Animals Fed Different Diets Values presented as mean±SEM. Bars that do not share a common letter are significantly different, within a mitogen ($p<0.05$). ConA:BBn control (n=13), BBn FF (n=15), BBdp control (n=15), BBdp FF (n=15).PMA & Iono:BBn control (n=8), BBn FF (n=10), BBdp control (n=12), BBdp FF (n=13).

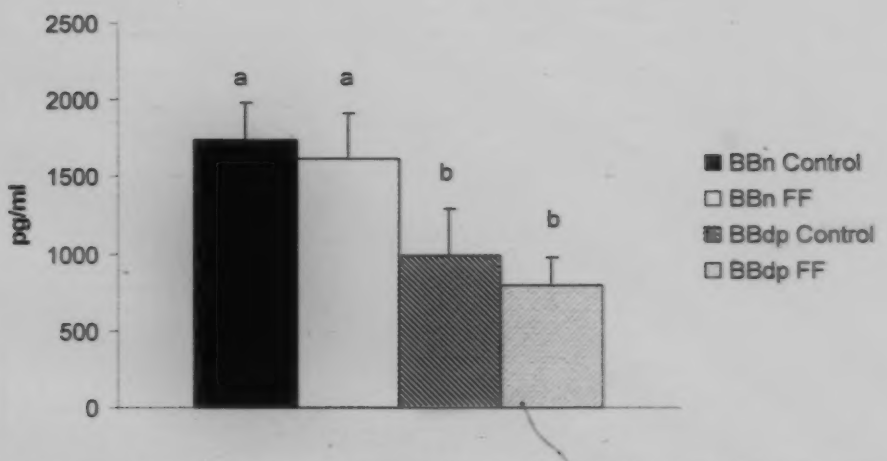


Figure 11 Mesenteric Lymph Nodes: ConA-Stimulated IFN-γ Production.

Values presented as mean±SEM. Bars that do not share a common letter are significantly different ($p<0.05$). BBn control (n=10), BBn FF (n=12), BBdp control (n=9), BBdp FF (n=9).

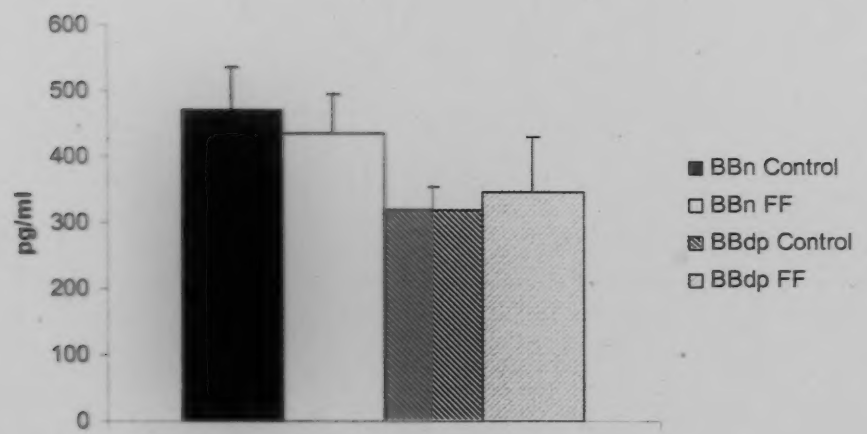


Figure 12 Mesenteric Lymph Nodes: ConA-Stimulated TNF-α Production.

Values presented as mean±SEM. Bars that do not share a common letter are significantly different ($p<0.05$). BBn control (n=14), BBn FF (n=16), BBdp control (n=7), BBdp FF (n=9).

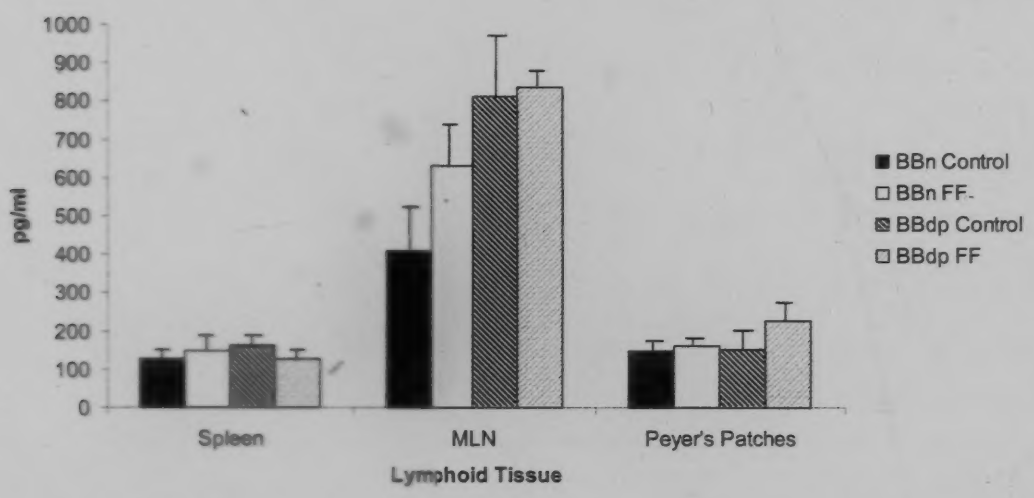


Figure 13 ConA-Stimulated TGF-β Production by cells from the Spleen, Mesenteric Lymph Nodes and Peyer's Patches.

Values presented as mean±SEM. Bars that do not share a common letter are significantly different, no significant differences found in TGF-β production in any groups ($p<0.05$). Peyer's Patches:BBn control (n=13), BBn FF (n=8), BBdp control (n=10), BBdp FF (n=7).

IgA-Bearing Lymphocytes in the Lamina Propria Region Within the Jejunum of 35-day-old BBn and BBdp Animals Fed Different Diets

In 35 day-old BBn animals, the expression of IgA within the lamina propria did not differ significantly between animals fed the control diet (58±6) and animals fed the FF diet (48±5) . However, BBdp animals fed the FF diet had a significantly higher number of IgA-bearing lymphocytes (88±7) in the lamina propria compared to all other groups (BBdp control 42±5) (P=0.0036) (Figure 14). Background staining was not detected when slides were stained in the absence of a primary antibody, nor when stained with an isotype-control, anti-pig CD45.

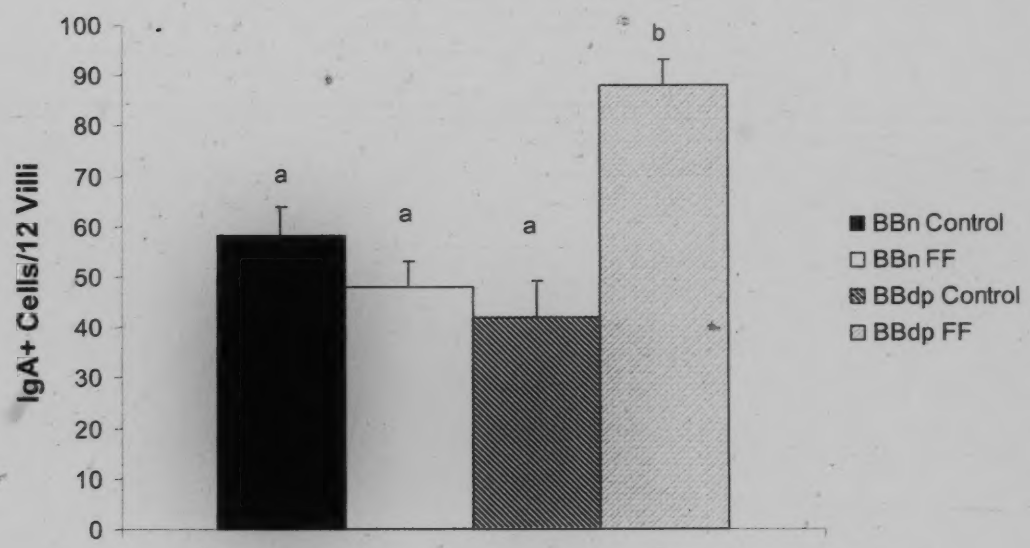


Figure 14 IgA-Bearing Cells in the Lamina Propria of Jejunal sections from BBn and BBdp Animals fed Different Diets.

Values presented as mean±SEM of number of IgA+ cells/12 villi (n=12/group).

B7.1 (CD80) Staining in Jejunal Sections of BBn and BBdp Animals.

Staining jejunal sections from BBdp an BBn animals with CD80/B7.1 did not result in consistent or measurable staining in the villus epithelium, however, in BBn animals, distinctive staining was found in the cryptic epithelium, at the base of the crypts of Leiberkuhn (Figure 15). The cells stained were Paneth cells as confirmed by examination of hematoxylin and eosin-stained slides (Figure 15). There was no significant difference in the absolute number of Paneth cells/ 9 crypts observed amongst groups (BBdp 35±3, BBn 39±2), as determined by hematoxylin and eosin staining (Figure 17). BBdp animals have significantly less CD80+ Paneth cells than BBn animals ($P<0.0001$) (Figure 17), but there was no effect of diet. Controls using a blocking peptide and staining in the absence of a primary antibody resulted in no background staining in the Paneth Cells.

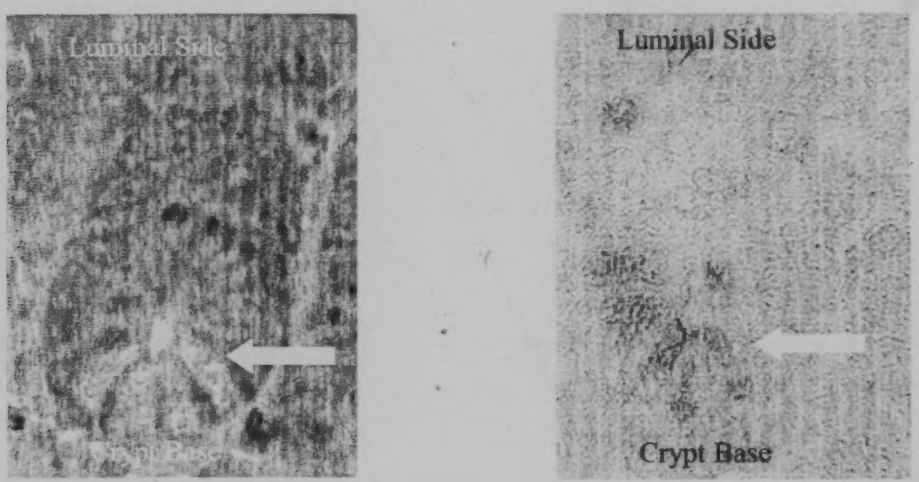


Figure 15 Stained Jejunal Crypts.

The figure on the left represents Hematoxylin-Stained stained jejunal crypt[1,2] . The figure on the right represents B7.1 Stained Jejunal[1,2].

[1]Paneth cells at the crypt base indicated by arrows
[2] Both samples from BBn animals fed control diet

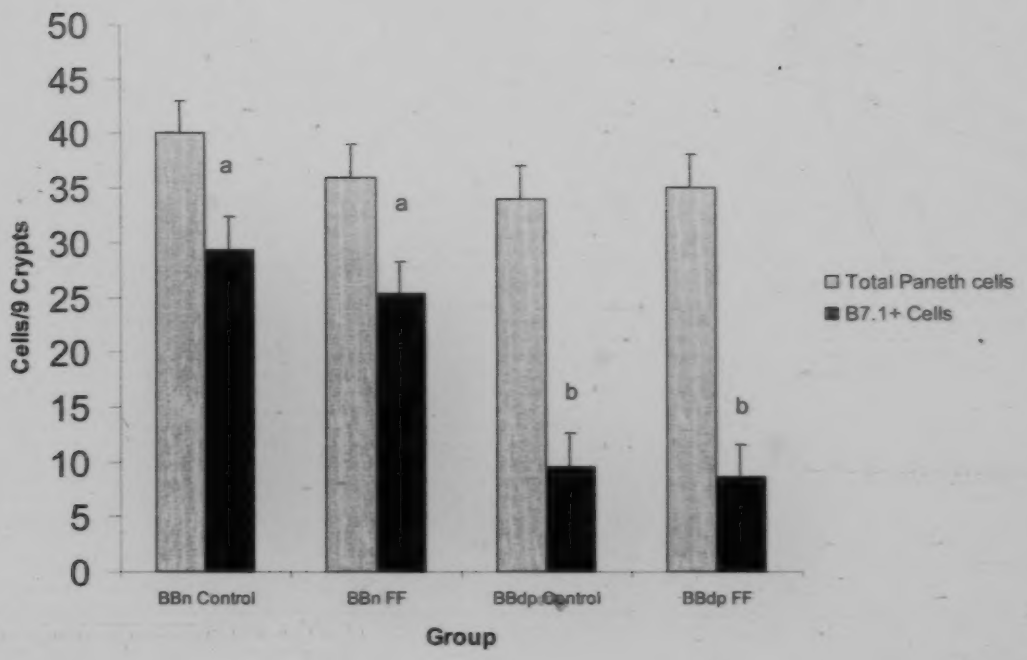


Figure 16 Prevelance of Paneth Cells and CD80+ cells per 9 Crypts in Jejunal Sections from BBdp and BBn Animals.

Values presented as mean±SEM. Bars of the same shade not sharing a common letter denotes significant differences amongst groups ($p<0.05$). (n=11/group).

A distinctive pattern of staining in the crypt in the area above which Paneth cells reside was observed. We measured the proportion of area stained in the lower third of the crypt above the Paneth cells, where the staining was present. No staining was observed in the villous epithelium above the crypts. The proportion CD80+ staining in the lower third of the crypts (expressed as mean±SEM of 9 crypts/sample) in BBdp rats was significantly greater than in BBn rats (P=0.0002) (Figure 17).

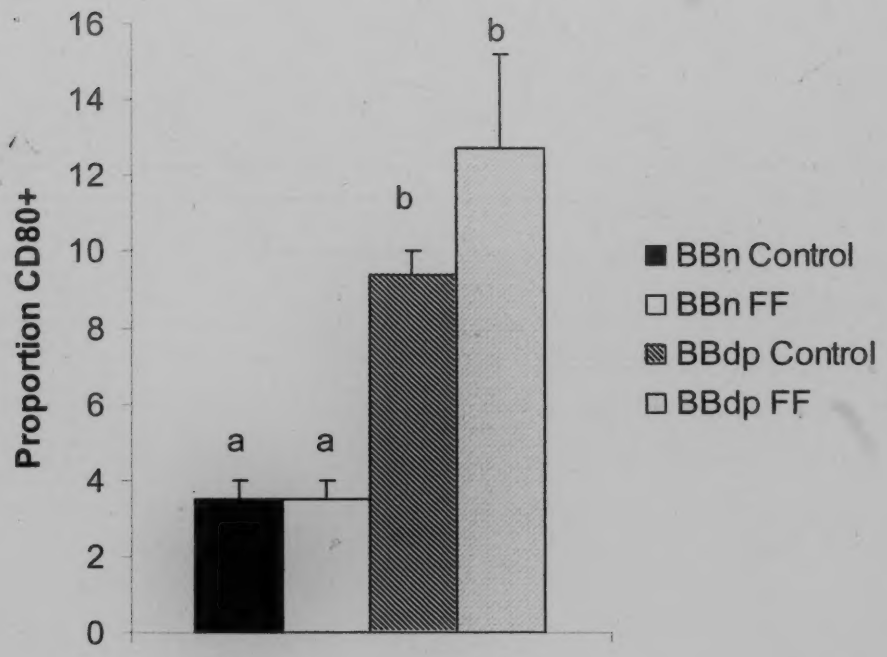


Figure 17 Proportion (%) of CD80+ staining in the lower third region of the crypt above the Paneth cells.

Bars represent mean±SEM (n=8/group). Bars that do not share the same letter are significantly different ($P<0.05$)

Immune Development: The Effect of Age and Strain on Immune Parametres in the BB rat

Characteristics at 21 and 35 Days of Age in BBn and BBdp Animals Fed Control Diet

At 21 days of age, BBn animals had a significantly greater number of MLN lymphocytes than BBdp animals ($P<0.0001$). All other characteristics measured did not differ significantly between BBn and BBdp animals at 21 days of age (Table 18). In BBn and BBdp animals fed control diet, there were significant differences found between 21 and 35 day old animals. Body weight, spleen weight and gut length were significantly higher in both BBn and BBdp animals at 35 days than at 21 days ($P<0.0021$). There was no significant effect of sex on body weights in these groups (Table 19). The number of splenocytes per gram spleen was lower in both 35-day-old BBn and BBdp animals than their 21-day-old counterparts ($P=0.0007$), while no difference was observed between strains.

Table 18 Animal Characteristics in BBn and BBdp Animals at 21 and 35 Days of Age[1,2]

Strain	BBn	BBn	BBdp	BBdp	Significance		
Age	21 days	35 days	21 days	35 days	Strain	Age	Interaction (strain*age
Characteristic							
Body Weight (g)	43 ± 1 (25)[a]	103 ± 3 (30)[b]	48 ± 2 (29)[a]	108 ± 3 (36)[b]	NS	P<0.0001	NS
Gut Length (cm)	56 ± 1 (23)[a]	81 ± 1 (26)[b]	55 ± 1 (27)[a]	80 ± 1 (24)[b]	NS	P<0.0001	NS
Spleen Weight (mg)	217 ± 15 (22)[a]	531 ± 18 (24)[b]	245 ± 25 (24)[a]	400 ± 24 (22)[c]	P<0.0001	P<0.0021	P<0.0001
# Splenocytes ($x10^6$)	87 ± 8 (24)[a]	154 ± 12 (25)[b]	82 ± 9 (24)[a]	109 ± 11 (24)[c]	P=0.0083	P<0.0001	P=0.042
# Splenocytes ($x10^6$)/ g spleen	451 ± 50 (22)[a]	263 ± 18 (22)[b]	358 ± 32 (23)[a]	274 ± 42 (17)[b]	NS	P=0.0007	NS
# MLN cells ($x10^6$)	5.6 ± 0.6 (25)[a]	22.5 ± 1.8 (25)[b]	2.7 ± 0.3 (26)[c]	6.7 ± 0.7 (25)[a]	P<0.0001	P<0.0001	P<0.0001

Table 19 Body Weights Separated by Sex

Strain	BBn		BBdp		Significance		
Age	21	35	21	35	Age	Strain	Sex
Characteristic							
Body Weight (g)	43 ± 3 (25)	103 ± 3 (30)	47 ± 3 (29)	108 ± 3 (36)	P<0.0001	NS	NS
Male	44 ± 5 (11)[a]	110 ± 4 (15)[b]	50 ± 4 (16)[a]	109 ± 4 (14)[b]	P<0.0001	NS	
Female	43 ± 4 (14)[a]	97 ± 4 (15)[c]	45 ± 4 (13)[a]	107 ± 3 (22)[cb]	P<0.0001	NS	

[1] Valies presented as mean±SEM (n)
[2] Within a row, values that do not share the same superscript are significantly different ($p<0.05$)

The Effect of Age and Strain on Lymphocyte Subsets in the Spleen from BBn and BBdp Animals Fed Control Diet

The proportion of T-cells (Ox19+, W3/13+, Ox8+, W325+) (Figure 18) was higher in 21-day old BBn than 21-day-old BBdp spleens ($P<0.0002$) (Table 20). There were higher proportions of W3/13+, Ox19+/W3/13+, and W3/25+ cells in the 35-day-old animals compared to the 21-day old animals in both BBn and BBdp groups ($P<0.0001$) (Table 20). In BBdp (but not BBn) animals, there was a significantly higher proportion of Ox19+ and Ox42+ lymphocytes at 35 days-of age than at 21-days-of-age ($P=0.0104$) (Figure 20). In BBn (but not BBdp) animals, there was a significantly lower proportion of Ox12+ lymphocytes in animals at 35-days-of-age compared to animals at 21-days-of-age ($P=0.012$) (Table 20, Figure 19).

The Effect of Age and Strain on Lymphocyte Subsets in the Mesenteric Lymph Nodes from BBn and BBdp Animals Fed Control Diet

Twenty-one-day old BBn animals had a significantly higher proportion of T-cell markers (Ox19+, W3/13+, Ox19+/W3/13+, Ox8+, and W3/25+ lymphocytes) than 21-day old BBdp animals ($P<0.0001$) (Table 21). The ratio of Ox8/W325 was lower in the BBn animals than the BBdp animals at both ages ($P=0.0006$). BBdp animals had a significantly higher percentage of Ox12+ and Ox42+ lymphocytes than BBn animals ($P=0.02$) (Table 21). Both BBn and BBdp animals showed a greater proportion of W313+ lymphocytes at 35 days compared to 21 days-of-age animals ($P=0.05$). The percentage of Ox8+ lymphocytes was lower in 35-day-old BBn animals than in the 21-day old animals ($P=0.02$). An Ox8 (CD8) lymphopenia was present in MLN at both ages in BBdp rats (Table 21).

Table 20 Spleen Lymphocytes: Effect of Age and Strain on Phenotype Distribution[1,2]

Strain	*BBn*		*BBdp*		*Significance*		
age	21 days	35 days	21 days	35 days	Age	Strain	Interaction (age*Strain)
Ox19+	23 ± 1 (14)[a]	29 ± 2 (16)[a]	10 ± 2 (11)[b]	22 ± 5 (7)[a]	P=0.0005	P=0.0002	NS
W313+	24 ± 2 (14)[a]	30 ± 2.5 (11)[b]	14 ± 2 (10)[c]	23 ± 3 (7)[a]	P=0.02	P<0.0001	NS
Ox19+/w3/13+	6 ± 0.7(14)[a]	22 ± 3 (13)[b]	5 ± 1 (11)[a]	11 ± 2 (7)[c]	P=0.0049	P<0.0001	NS
Ox8+	14 ± 2 (14)	14 ± 1 (15)	7 ± 1 (8)	9 ± 1 (11)	NS	P<0.0001	NS
W325+	17 ± 2 (13)	22 ± 1 (14)	6 ± 1 (9)	11 ± 2 (11)	P=0.001	P<0.0001	NS
Ox12+	33 ± 2 (15)[a]	22 ± 2 (13)[b]	36 ± 2 (11)[a]	37 ± 3 (11)[a]	P=0.036	P<0.0001	P=0.104
W3/25:Ox8	1.3 ± 0.2	1.7 ± 0.1 (13)	1 ± 0.2 (8)	1.4 ± 0.2 (10)	NS	NS	NS
Ox42+	7 ± 1 (11)[a]	8 ± 1 (13)[a]	5 ± 1(8)[a]	13 ± 2 (11)[b]	P=0.02	NS	P=0.012
323+	8 ± 1.5 (16)	9 ± 1 (14)	5 ± 0.6 (8)	8 ± 2 (7)	NS	NS	NS

Table 21 MLN Lymphocytes: Effect of Age and Strain of Phenotype Distribution[1,2]

Strain	*BBn*		*BBdp*		*Significance*		
age	21 days	35 days	21 days	35 days	Age	Strain	Interaction (age*Strain)
Ox19+	70 ± 3 (11)[a]	73 ± 2 (13)[a]	26 ± 4 (7)[b]	25 ± 1 (14)[b]	NS	P<0.0001	NS
W313+	68 ± 3 (11)[a]	74 ± 3 (14)[b]	15 ± 2 (10)[c]	27 ± 2 (11)[d]	P=0.05	P<0.0001	NS
Ox19+/w313+	63 ± 3 (11)[a]	62 ± 3 (15)[a]	13 ± 1 (9)[b]	20 ± 1 (14)[b]	NS	P<0.0001	NS
Ox8+	24 ± 1 (12)[a]	20 ± 1 (17)[b]	5 ± 1 (9)[c]	4 ± 1 (12)[c]	P=0.02	P<0.0001	P=0.04
W325+	54 ± 1 (12)[a]	56 ± 2 (12)[a]	18 ± 2(10)[b]	20 ± 2 (16)[b]	NS	P<0.0001	NS
Ox12+	19 ± 1 (15)[a]	15 ± 1 (15)[a]	47 ± 2 (11)[b]	45 ± 3 (16)[b]	NS	P<0.0001	NS
W325:Ox8	2.3 ± 0.1 (12)[a]	2.7 ± 0.1 (11)[a]	4.2 ± 0.7 (7)[b]	7.2 ± 1.8 (8)[b]	NS	P=0.0006	NS
Ox42+	5 ± 1 (10)[a]	5 ± 1 (15)[a]	6 ± 1 (11)[a]	9 ± 2 (11)[b]	NS	P=0.02	NS

[1] Values presented as proportion (%) of total gated cells; mean ± SEM (n)
[2] Within a row, values that do not share the same superscript are significantly different ($p<0.05$)

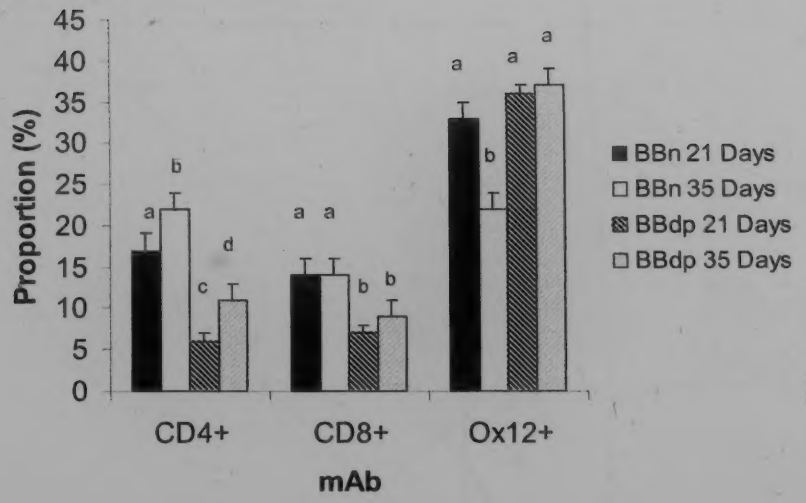


Figure 18 Spleen Lymphocytes: T-cell (CD4 and CD8) and B-cell Distribution (Ox12) in BBdp and BBn Animals at 21 and 35 days of age.

Values presented as mean±SEM. Bars within a mAB that do not share a common letter are significantly different within an antibody ($p<0.05$).

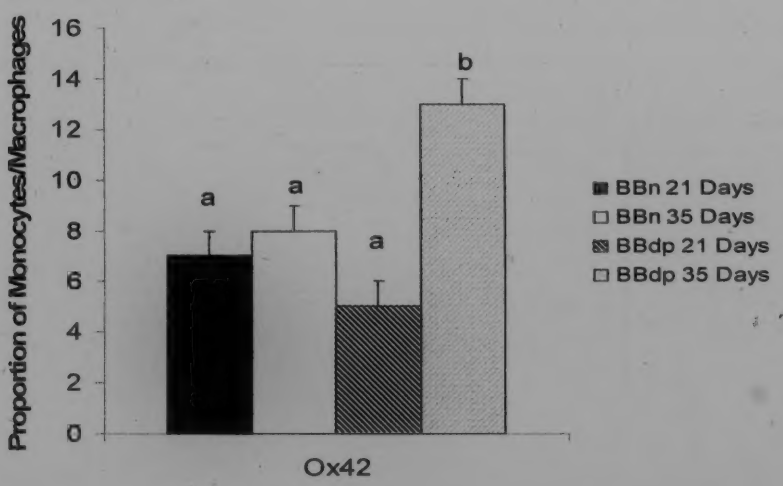


Figure 19 Spleen Lymphocytes: Proportion of Ox42+ cells (Macrophages/monocytes)

BBn control (n=11) BBn FF (n=13) BBdp control (n=8) BBdp FF (n=10). Values presented as mean±SEM. Bars within a mAB that do not share a common letter are significantly different ($p<0.05$).

Spleen Lymphocytes: Rate of ^{3}H-thymidine Uptake in 21 and 35 Day-Old BBn and BBdp Animals Fed Control Diet

Splenocytes in absence of a mitogen from 21-day old BBn animals had significantly lower ^{3}H-thymidine uptake (DPM) compared to 21-day-old BBdp animals (P=0.03) (Table 22). Splenocytes from 21-day-old BBn animals had a significantly greater SI when stimulated with PMA and Ionomycin than 21-day-old BBdp animals (P<0.0001) (Table 22) (Figure 21). Twenty-one-day-old BBdp animals had a significantly higher rate of ^{3}H-Thymidine uptake than BBn animals of the same age when splenocytes were incubated with NZMP and soy protein (P=0.0002). A higher SI was observed in 35-day- old BBn animals but not BBdp animals when splenocytes were stimulated with ConA (P<0.0001).This age-related increase in response to ConA was not observed with cells from BBdp animals. Incubation with NZMP resulted in a higher rate of ^{3}H-thymidine uptake in BBn animals at 35 days compared to 21 days-of-age, while no difference was observed in the BBdp animals (P=0.0002). Splenocytes from 21-day-old BBdp animals incubated with soy protein showed a greater rate of ^{3}H-thymidine uptake than at 35-day-old BBdp animals while no difference in rate of ^{3}H-thymidine uptake was observed in the BBn animals (P<0.0001).

Spleen Lymphocytes: Mitogen-Stimulated Cytokine Production from BBn and BBdp Animals 21 and 35 days of age Fed Control Diet

At 21 days of age, mitogen stimulation of splenocytes from BBdp and BBn animals resulted in a number of differences. At 21 days, BBn animals had a significantly higher ConA stimulated IL-2 and IL-10 production (P<0.0001) (Figure 22 and 23, respectively), a higher PMA & Iono stimulated IL-2 (P<0.0001) (Figure 22) and IL-10 (P=0.005) (Figure 22) and IL-4 (P<0.0001) production than BBdp animals (Table 23). For both strains, a significant effect of age was observed in the production of the following; unstimulated IL-10 (P<0.0001), ConA-stimulated IL-10 (P=0.0002), PMA & iono-stimulated IL-2 (P<0.0001) (Figure 22) and IL-10 production by cells incubated with NZMP (P<0.0001) and soy proteins (P=0.0007) (Table 23) (Figure 24). In all cases where the effect of age was significant, cytokine production at 35 days was higher than at 21 days of age. The production of PMA & Iono-stimulated IFN-γ and ConA-stimulated TGF-β did not differ amongst age and strain (Table 23).

Table 22 Spleen Lymphocytes: Rate of ^{3}H-thymidine Uptake in 21 and 35 Day-Old BBn and BBdp Animals Fed Control Diet

Strain		*BBn*		*BBdp*		*Significance*		
age		21 days	35 days	21 days	35 days	Age	Strain	Age*Strain
Mitogen								
Unstimulated	*DPM*[3]	970 ± 168 (21)[a]	1756 ± 248 (27)[b]	1517 ± 162 (14)[b]	1933 ± 197 (16)[b]	P=0.003	NS	NS
ConA	*DPM*	6369 ± 3412 (14)[a]	100072 ± 24220 (8)[b]	2618 ± 1179 (7)[a]	4833 ± 1665 (9)[a]	P<0.0001	P=0.0002	P=0.0005
	SI[4]	6 ± 2 (14)[a]	39 ± 6 (8)[b]	2 ± 1(7)[a]	4 ± 2 (9)[a]	P<0.0001	P<0.0001	P<0.0001
PMA& Iono	*DPM*	28782 ± 6496 (9)[a]	51529 ± 12820 (16)	4417 ± 529 (13)[b]	9998 ± 467 (16)[b]	NS	P<0.0001	NS
	SI	17 ± 3 (9)[a]	19 ± 2 (16)[a]	3 ± 1 (13)[b]	5 ± 1 (16)[b]	NS	P<0.0001	NS
Hydrolysed	*DPM*	515 ± 128 (13)[a]	1083 ± 184 (16)[b]	1519 ± 178 (9)[b]	1722 ± 293 (6)[b]	NS	P=0.0002	NS
Casein (NZMP)	*SI*	0.8 ± 0.1 (13)	0.9 ± 0.1 (16)	0.9 ± 0.1 (9)	1.1 ± 0.1 (6)	NS	NS	NS
SOY	*DPM*	422 ± 99 (8)[a]	575 ± 51 (10)[a]	1614 ± 265 (6)[b]	608 ± 267 (4)[a]	NS	P<0.0001	NS
	SI	1.1 ± 0.1 (8)	0.9 ± 0.06 (10)	0.9 ± 0.2 (6)	0.8 ± 0.1(4)	NS	NS	NS

[1] Values presented as mean±SEM (n)
[2] Within a row, values that do not share the same superscript are significantly different ($p<0.05$)
[3] DPM (Decays per minute)
[4] SI (stimulation index calculated as DPM stimulated/DPM unstimulated

Table 23 Spleen Lymphocytes: The Effect of Age and Strain of Mitogen-Stimulated Cytokine Production [1,2]

	Strain	*BBn*		*BBdp*		*Significance*		
	Age	21	35	21	35	age	strain	age*strain
Mitogen	Cytokine							
Unstimulated	IL-10	158 ± 18 (15)^{a}	399 ± 57(14)^{b}	122 ± 15 (14)^{a}	291 ± 29 (14)^{c}	p<0.0001	p=0.03	NS
ConA	IL-2	2491 ± 154 (11)^{a}	2420 ± 324 (8)^{a}	668 ± 128(8)^{b}	393 ± 64 (8)^{b}	NS	p<0.0001	NS
	IL-10	506 ± 53(13)^{a}	653 ± 29 (10)^{b}	272 ± 34 (12)^{c}	471 ± 43(10)^{a}	p=0.0002	p<0.0001	NS
	TGF-β	217 ± 42 (6)	128 ± 20 (12)	189 ± 33 (10)	161 ± 23 (10)	NS	NS	NS
PMA & Iono	IL-2	2597 ± 370 (10)^{a}	4871 ± 191 (6)^{b}	556 ± 75 (14)^{c}	1782 ± 260(9)^{d}	p<0.0001	p<0.0001	p=0.036
	IFN-γ	954 ± 185 (13)	591 ± 95 (8)	899 ± 154 (12)	890 ± 192 (8)	NS	NS	NS
	IL-10	729 ± 215 (8)^{a}	690 ± 207 (9)^{a}	150 ± 17 (14)^{b}	476 ± 51 (9)^{a}	NS	p=0.005	NS
	IL-4	50 ± 6(9)^{a}	35 ± 6(10)^{a}	15 ± 2 (13)^{b}	21 ± 6(10)^{b}	NS	p<0.0001	NS
NZMP	IL-10	368 ± 78 (11)^{a}	469 ± 51 (17)^{a}	86 ± 12(11)^{c}	224 ± 37(11)^{d}	p=0.04	p<0.0001	NS
SOY	IL-10	175 ± 43 (12)^{a}	254 ± 30 (7)^{b}	92 ± 10(10)^{a}	280 ± 33 (8)^{b}	p=0.0007	NS	NS

[1]Values presented as pg/ml mean±SEM (n)

[2] Within a row, values that do not share the same superscript are significantly different ($p<0.05$)

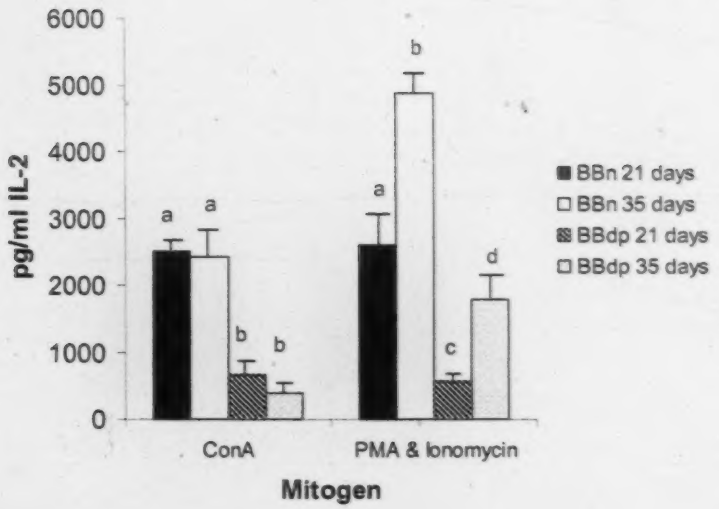


Figure 20 Spleen Lymphocytes: Mitogen-Stimulated IL-2 Production by 21 and 35-day -old BBn and BBdp Animals. ConA: BBn control (n=11) BBn FF (n=8) BBdp control (n=8) BBdp FF (n=8). PMA & Iono: BBn control (n=10) BBn FF (n=6) BBdp control (n=14) BBdp FF (n=9). Values presented as mean±SEM. Bars that do not share a common letter are significantly different ($p<0.05$).

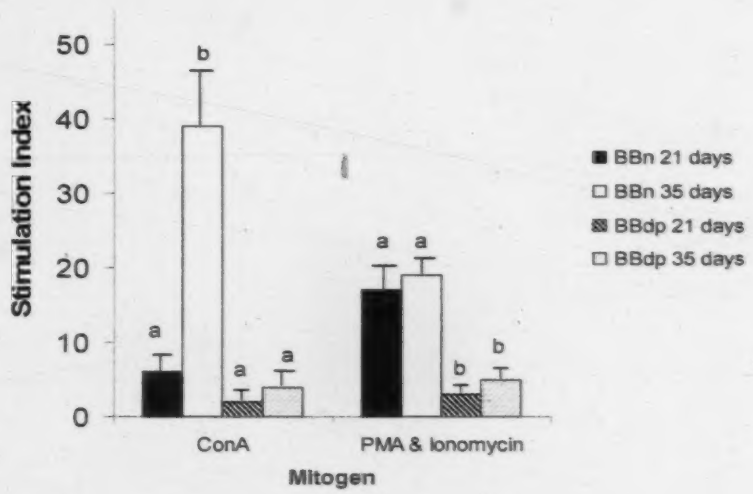


Figure 21 Spleen Lymphocytes: Mitogen-Stimulated ^{3}H-Thymidine Uptake by 21 and 35-day -old BBn and BBdp Animals. ConA:BBn control (n=14) BBn FF (n=8) BBdp control (n=7) BBdp FF (n=9). PMA & Iono: BBn control (n=9) BBn FF (n=16) BBdp control (n=13) BBdp FF (n=16). Values presented as mean±SEM. Bars that do not share a common letter are significantly different ($p<0.05$).

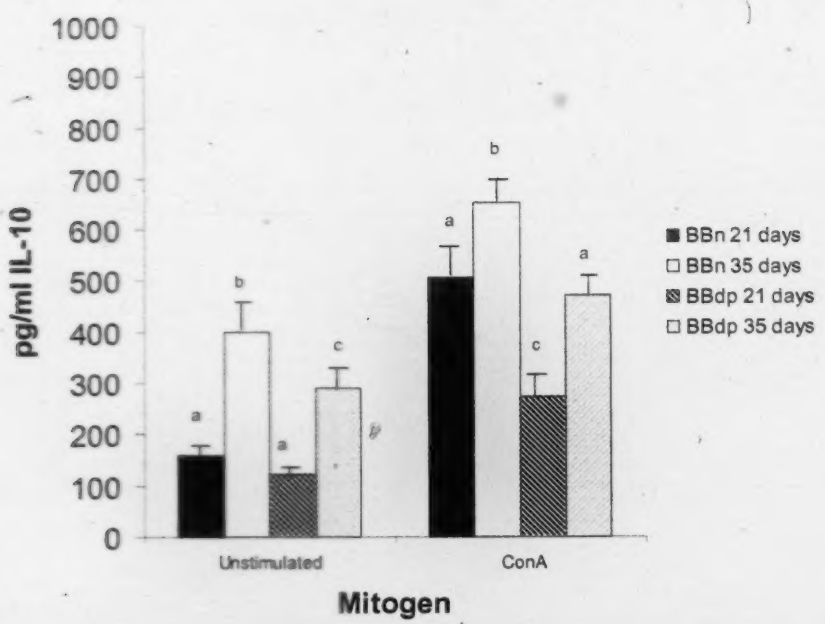


Figure 22 Spleen: Mitogen-stimulated IL-10 production in 21 and 35 day old BBdp and BBn animals Unstimulated: BBn control (n=15) BBn FF (n=14) BBdp control (n=14) BBdp FF (n=14).ConA: BBn control (n=13) BBn FF (n=10) BBdp control (n=12) BBdp FF (n=10).Values presented as mean±SEM. Bars that do not share a common letter are significantly different ($p<0.05$).

Mesenteric Lymph Node Lymphocytes: Rate of ^{3}H-thymidine Uptake in 21 and 35 Day-Old BBn and BBdp Animals Fed Control Diet

MLN lymphocytes from 21-day-old BBn animals stimulated with both ConA, and PMA plus Ionomycin had a significantly higher rate of ^{3}H-thymidine uptake and SI than in 21-day-old BBdp animals ($P<0.0001$) (Table 24). Lymphocytes stimulated with ConA showed a significant effect of age ($P=0.015$), with both BBn and BBdp animals having a greater rate of ^{3}H-thymidine uptake than 21-day-old rats. However, only the SI from ConA stimulated BBn cells from the 21-day-old BBn animals was significantly lower than the 35-day-old BBn animals ($P=0.023$) (Table 24) (Figure 23). This effect of age on the SI by ConA-stimulated cells was not seen in BBdp animals. At 21 days-of-age the rate of ^{3}H-thymidine uptake and SI to PMA + Ionomycin was significantly higher in BBn compared to BBdp MLN lymphocytes ($P<0.0001$). The response to PMA and Ionomycin increased with age for BBdp lymphocytes (but not BBn) to a rate of ^{3}H-thymidine uptake and SI not different from 35-day-old BBn animals ($P=0.003$). There was no effect of age or strain on the response of MLN lymphocytes to NZMP or soy.

Mesenteric Lymph Node Lymphocytes: The Effect of Age and Strain on Mitogen-Stimulated Cytokine Production

There was no effect of age or strain on the following parameters: unstimulated IL-10 production, ConA-stimulated TNF-α and TGF-β production, and the production of IL-10 by cells stimulated with the baby formula protein NZMP (Table 25). ConA-stimulated IFN-γ production was significantly higher in 21-day-old BBn animals than 21-day-old BBdp rats ($P<0.0001$) (Table 25, Figure 24). Additionally, while there was no effect of age on ConA-stimulated IFN-γ production from MLN cells in BBdp animals, cells from BBn animals produced more IFN-γ after ConA stimulation at 21 days-of-age than at 35 days-of-age ($P=0.04$).

IgA+ cells in the Lamina Propria of the Jejunum from 21 and 35-day-old BBn and BBdp Animals Fed Control Diet

IgA expression within the lamina propria did not differ between 21-day old BBn (n=9) and 21-day old BBdp (n=10) animals but was very low (~1 IgA+ cell/12 villi, data not illustrated). Thus, there was a significant effect of age on the expression of IgA in the lamina propria in BBn and BBdp animals fed control diet for 35-day-old animals was 58± 6 and 42± 5, respectively (n=12) ($P<0.0001$).

B7.1 (CD80) in Jejunal Sections from 21 and 35-day-old BBdp and BBn animals Fed Control Diet

Paneth Cells stained positive for CD80 in 21-day-old BBn animals and BBdp animals. The number of CD80+ cells per 9 crypts did not differ between BBdp (2±1 n=9) and BBn (4±2 n=9) animals at 21 days. In both strains, the number of CD80+ cells was significantly higher at 35 days than at 21 days ($P<0.0001$). However, the increase in CD80+ cells was significantly greater in BBn (25±2 n=12) animals than in BBdp (10±3 n=12) animals ($P=0.0032$) (Figure 25). The absolute number of Paneth cells at 35 days was not different between groups. The proportion of positive staining for CD80 in the lower third of the crypt, above the region where Paneth cells are found was not different between BBdp and BBn rats at 21 days of age. The proportion of stained area was significantly lower at 35 days of age than at 21 days of age in BBn rats ($P=0.001$), but not in BBdp rats.

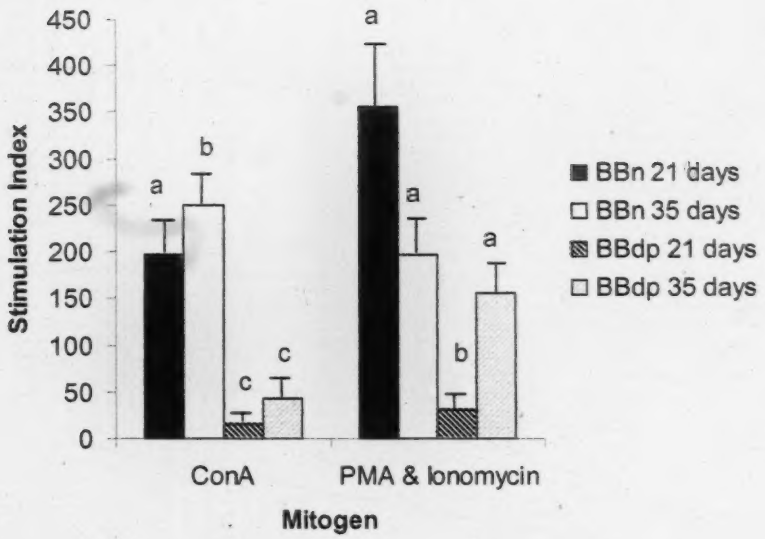


Figure 23 MLN: The mitogen-stimulated ^{3}H-thymidine uptake in BBn and BBdp rats at 21 and 35 days of age. BBn control (n=11) BBn FF (n=13) BBdp control (n=9) BBdp FF (n=16).PMA & Iono: BBn control (n=7) BBn FF (n=8) BBdp control (n=7) BBdp FF (n=12). Values presented as mean±SEM. Bars that do not share a common letter are significantly different within a mitogen ($p<0.05$).

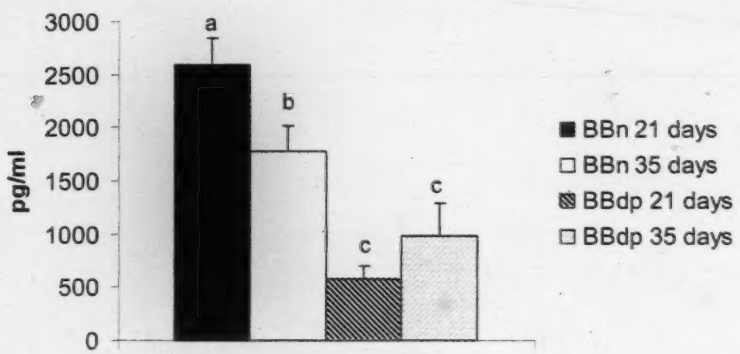


Figure 24 MLN: The ConA-Stimulated production of IFN-γ from BBn and BBdp animals at 21 and 35 days of age BBn control (n=8) BBn FF (n=10) BBdp control (n=4) BBdp FF (n=9). Values presented as mean±SEM. Bars that do not share a common letter are significantly different within a mitogen ($p<0.05$).

Table 24 Mesenteric Lymph Nodes: The Effect of Age and Strain on ^{3}H-Thymidine Incorporation

Strain		*BBn*		*BBdp*		*Significance*		
Age		21 days	35 days	21 days	35 days	Age	Strain	Interaction (Age*Strain)
Mitogen								
Unstimulated	*DPM*	321 ± 55 (18)	558 ± 196 (19)	409 ± 99 (11)	528 ± 92 (20)	NS	NS	NS
ConA	*DPM*	72979 ± 17630 (11)[a]	136518 ± 23874 (13)[b]	6033 ± 2974 (11)[c]	27856 ± 7742 (16)[d]	P=0.015	P<0.0001	NS
	SI	196 ± 48 (11)[a]	249 ± 36 (13)[b]	15 ± 5 (9)[c]	43 ± 11 (16)[c]	NS	P<0.0001	NS
PMA& Iono	*DPM*	92676 ± 25083 (7)[a]	66482 ± 15544(8)[ab]	10979 ± 3455 (7)[b]	70142 ± 18043 (12)[a]	NS	NS	P=0.034
	SI	356 ± 86 (7)[a]	196 ± 28 (8)[a]	31 ± 7 (7)[b]	155 ± 29[a] (12)	NS	P=0.0002	P=0.003
NZMP	*DPM*	264 ± 39 (11)	784 ± 297 (16)	264 ± 39 (11)	526 ± 212 (6)	NS	NS	NS
	SI	1.1 ± 0.1 (11)	1.2 ± 0.1 (16)	1.1 ± 0.1 (11)	1.2 ± 2.1 (6)	NS	NS	NS

Table 25 Mesenteric Lymph Nodes: The Effect of Age and Strain on Mitogen-Stimulated Cytokine Production

Strain		*BBn*		*BBdp*		*Significance*		
Age		21 days	35 days	21 days	35 days	Age	Strain	age*strain
Mitogen	*Cytokine*							
Unstimulated	*IL-10*	22 ± 7 (8)	54 ± 36 (9)	20 ± 9 (5)	144 ± 42 (6)	NS	NS	NS
ConA	*IFN-γ*	2587 ± 262 (8)[a]	1782 ± 242 (10)[b]	577 ± 119 (4)[c]	987 ± 299 (9)[c]	P=0.04	P<0.0001	NS
	TNF-α	447 ± 93 (7)	469 ± 67(14)	281 ± 79 (3)	320 ± 34(7)	NS	NS	NS
	TGF-β	701 ± 164 (8)	408 ± 113 (8)	417 ± 103 (4)	810 ± 168 (8)	NS	NS	NS
NZMP	*IL-10*	65 ± 7 (7)	151 ± 49 (12)	48 ± 22 (4)	115 ± 24(10)	NS	NS	NS

[1]Values presented as pg/ml mean±SEM (n)

[2] Within a row, values that do not share the same superscript are significantly different ($p<0.05$)

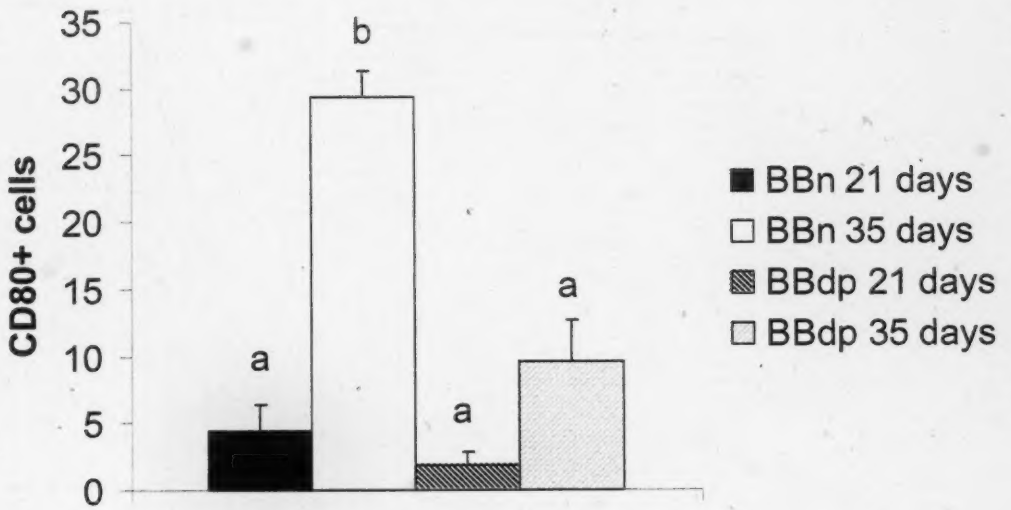


Figure 25 CD80+ Paneth cells at crypt base in BBn and BBdp rats at 21 and 35 days of age.

Bars that do not share the same letter are significantly different ($P<0.05$). Vlaues expressed as the proportion mean ± SEM. BBn and BBdp 21 days (n=8) BBn and BBdp 35 days (n=11).

Chapter 5:Discussion

Weaning, the transition from dam's milk to adult food (Van der Heijden *et al*, 1990), is associated with a number of changes in morphology (Cummins *et al*, 1988b) and immune activation in the intestine and periphery (Cummins *et al*, 1988a) (Masjedi *et al*, 1999). It is thought that the development of the immune system is antigen-driven and the presence of intestinal bacteria can facilitate maturation of immune function in both the intestine and periphery (Bjorksten, 1999). Luminal bacteria are directly modified by dietary fibre (Buddington *et al*, 2002) (Bouhnik *et al*, 1996) (Le Blay *et al*, 1999) and by infant-feeding practices (Harmsen *et al*, 2000). Since early diet can modify resident bacteria, and this can drive immune development, it is possible that weaning to a diet containing fermentable fibres can influence diabetes outcome via its effects on immune development after weaning.

Previously, it has been shown that BBdp animals respond differently than BBn animals to a undefined, cereal-based weaning diet (Reimer *et al*, 1998) (Butler S, 1999). We hypothesized that feeding a semi-purified, casein-based diet with fermentable fibre (FF) would affect measures of immune function differently in young BBdp animals than in BBn animals. FF feeding compared to cellulose feeding resulted in a longer gut length (Figure 2), and a higher proportion of CD8+ cells (Figure 4) in the Peyer's Patches, and a higher PMA & Iono-stimulated uptake of ^{3}H-Thymidine by both splenocytes (Figure 5) and MLN cells (Figure 10) in BBn animals but not BBdp animals.

Gut Length

The increase in gut length, after feeding fermentable fiber, suggests that the enterotrophic effect of chow, previously reported (Reimer *et al*, 1998), is due to the presence of FF in the diet. Feeding diets high in FF have been reported to increase villus height, increase intestinal proglucagon mRNA and intestinal glucose transporters in healthy dogs (Massimino *et al*, 1998). As proglucagon mRNA encodes for the enterotrophic hormone GLP-2, FF could mediate it's enterotrophic effect through an increased secretion of this hormone (Jasleen *et al*, 2002). Additionally, lower levels of colonic proglucagon mRNA have been reported in the BBdp rat compared to the BBn rat fed a purified, casein-based diet (Reimer *et al*, 1998). Similar to the observation in our study by feeding FF, feeding chow diet to BBn rats resulted in a lower colonic proglucagon mRNA compared to feeding a semi-purified diet, while diet did not affect proglucagon mRNA levels in BBdp rats (Reimer *et al*, 1998). This suggests that BBdp rats do not

produce sufficient GLP-2, or have an abnormal GLP-2 receptor expression in the gut. As a result of this, BBdp animals may not be able to increase gut length in response to dietary FF.

The longer gut in FF-fed BBn animals suggests that the diet provided an immunological challenge, as the immune system is involved in the growth and development of the small intestine at weaning (Cummins *et al*, 1989;Thompson *et al*, 1996). Antibodies administered to block the IL-2 receptor in rats resulted in decreased crypt depth compared to untreated rats (Thompson *et al*, 1996). Administration of cyclosporin A, an immunosuppressant, resulted in decreased villus height, however, gut length was not reported in this study (Cummins *et al*, 1989). BBn animals fed FF had a higher rate of ^{3}H-thymidine uptake by spleen and MLN cells after PMA & Ionomycin stimulation (Figure 4) than control-fed BBn rats, suggesting that there is a higher state of immune activity after lymphocyte activation.

γδ T-cells are known to produce growth factors involved in epithelial cell homeostasis, such as keratinocyte growth factor (Boismenu & Havran, 1994). Mice that are γδ T-cell deficient experience reduced intestinal growth (Komano *et al*, 1995). Intestinal γδ T-cells are CD8+ (Salerno & Dieli, 1998). The deficiency of intraepithelial γδ T-cells reported in BBdp rats (Ramanathan *et al*, 2002) might have also contributed to reduced intestinal growth. It is unknown whether the expected changes in the microflora, and subsequent production of SCFA's associated with feeding FF, affected the secretion of growth factors by IEL. However, it has been shown that feeding FF can alter the proportion of CD8+ IEL cells in the small intestine (Field *et al*, 1999b) (Ishizuka & Tanaka, 2002;Nagai *et al*, 2000), cecal mucosa (Ishizuka & Tanaka, 2002) and colorectum (Nagai *et al*, 2000). Consistent with these studies, we found an increase in the proportion of CD8+ cells in the Peyer's Patches of the small intestine of non-diabetic animals in response to FF feeding (Table 13). This suggests that in normal animals, the recruitment of more CD8+ cells (γδ T-cells) to the gut after FF feeding might have contributed to an increase in gut growth.

IgA+ Cells in the Lamina Propria

FF feeding compared to cellulose feeding resulted in a higher proportion Ox12+ B-cells in the Peyer's Patches (Figure 4), and a higher presence of IgA-bearing cells in the lamina propria (LP) of jejunal sections from BBdp but not BBn animals at 35 days of age (Figure 14). As hypothesized, feeding FF resulted in a measurable difference between BBn and BBdp animals in the gut immune system. Previously, it was shown that chow fed BBdp rats had a significantly

higher number of IgA + cells in the LP, while diet did not alter the expression of IgA+ cells in the LP of BBn rats (Butler S, 1999). The above results confirm the hypothesis that the fermentable fibre portion of chow is responsible for the increase in IgA+ cells in the jejunal lamina propria. Feeding different fibres has been reported to alter the expression of IgA-bearing cells in the gut. Feeding gum arabic, glucomannin, celfur, lactulose and curdlan, resulted in an increase in IgA+ cells in cecal and small intestinal mucosa of rats (Kudoh *et al*, 1998;Kudoh *et al*, 1999). A limitation to measuring IgA+ cells is that the presence of IgA-bearing cells in the lamina propria does not indicate IgA secretion into the lumen or secretory capacity of individual cells, but measures the number of cells that have likely been exposed to antigen in the gut and have migrated to the lamina propria. BB rats have many immune abnormalities (Field, 1995), but effects on IgA production and secretion have not been documented.

Germ-free mice have reduced amounts of IgA-producing cells in the intestine compared to conventional mice (Crabbe *et al*, 1968), suggesting that the number of IgA-bearing cells in the LP is antigen-dependent. Thus, an increased number of IgA+ cells in the lamina propria of BBdp rats after FF feeding, but not BBn rats, is suggestive of an increased exposure to antigen by cells in the Peyer's Patches. This could be due to a higher antigen uptake by the Peyer's Patches or to higher exposure caused by greater intestinal permeability (Meddings *et al*, 1999). The expected increase in gut microflora by feeding FF could also contribute to a greater antigenic load in the small intestine. In addition to a greater antigenic load, shifts in bacterial composition after FF feeding might contribute to an increased exposure to antigen in the gut or differences in immune activation. It is known that different strains of LAB have different effects on lymphocytes when monoassociated with a germ-free animal (Cross *et al*., 2001). Increased antigen exposure/uptake by Peyer's Patches would result in immune activation, providing a possible mechanism by which diet may alter the development of diabetes in this model.

Interestingly, the proportion of Ox12+ cells (B-cells) was significantly higher in the Peyer's Patches from BBdp animals fed the FF diet (Figure 4). This is consistent with the higher number of IgA+ cell in the LP. B-cells activated in the Peyer's Patches clonally expand in the germinal centres present within the Peyer's Patches (Kagnoff, 1993), and eventually migrate to the lamina propria, where they secrete IgA that transverses the epithelial lining and is secreted into the lumen as sIgA (Kagnoff, 1993). A key regulator in the committance to IgA secretion by B-cells is the cytokine TGF-β, which is produced by T_h3 CD4+ cells in the Peyer's Patches (Mayer, 2000). Once activated, T_h3 cells produce TGF-β, which induces class switching usually

from an IgG or an IgM-producing B-cell to an IgA producing B-cell (Mayer, 2000). The present study did not find any difference amongst groups in the ability of Peyer's Patch lymphocytes to produce TGF-β when stimulated by ConA by lymphocytes from the Peyer's Patches (Figure 13), or MLN. This is somewhat unexpected, given the marked reduction in T-cells in the Peyer's Patches of BBdp animals and suggests a higher production of this cytokine on a T cell basis.

Mitogen-stimulated ^{3}H-Thymidine Uptake and Cytokine Production

PMA & Ionomycin-stimulated ^{3}H-thymidine uptake was higher in splenocytes and MLN cells from BBn rats fed FF compared to control. The mechanism by which feeding FF could improve the stimulation response of lymphocytes to PMA & Ionomycin is not known. PMA & Iono activates Protein Kinase C in combination with an increase in intracellular calcium in most immune cell types, resulting in a cascade of events that lead to cell proliferation (Field, 1995). This pathway is a ubiquitous cell-signaling pathway. Diet differences observed after PMA & Iono stimulation of lymphocytes but not ConA, a mitogen that targets the lectin receptor on, or near, the TCR, suggests that the mechanism of increased ^{3}H-thymidine uptake by lymphocytes after FF feeding may be T cell independent. In further support of this, PMA & ionomycin-stimulated IL-2 production was not different between diets (Figure 5). IL-2 is produced by T cells and the amount of IL-2 produced by BBn cells did not differ between diets (Figure 6). This suggests that the effect of FF on the response to PMA + Iono may be mediated via other cell types. Diet did not affect PMA & Ionomycin-stimulated response by BBdp immune cells, supporting our hypothesis that lymphocytes from BBdp rats are not responding normally to luminal signals.

Regardless of disease susceptibility, feeding FF affected the mitogen-stimulated production of T_h2 cytokines in splenocytes increased IL-10 production in response to Con A and decreased IL-4 in response to PMA & Iono (Figure 9 and 8, respectively). The cytokine microenvironment in the gut and periphery, or the ability to respond to mitogens after FF feeding has not been well-characterized in normal animals. One study reported no difference in colonic IL-4 mRNA in mice after feeding fructo-oligosaccharides (Bassonga *et al*, 2001). However, this did not measure the ability of immune cells to produce IL-4. The functions of IL-10 and IL-4 however, have been extensively studied. IL-10 is able to modulate the immune response by inhibiting T-cell proliferation and the production of T_h1 cytokines (Taga & Tosato, 1992; Taga *et al.*, 1993). IL-10 also enhances the production of immunoglobulins by human B-cells when

added to culture (Armitage et al., 1993). IL-4 plays a role in mediating the differentiation of naïve T-cells towards a T_h2 specificity and like IL-10, suppresses T_h1 responses (Nelms et al., 1999). In addition, IL-4 can influence immunoglubulin class switching towards IgE, which is involved in allergy responses (Nelms et al., 1999). An increased production of IL-10 after lymphocyte activation could indicate a down-regulation of the T_h1 response after FF feeding. It has been found that IL-10 can inhibit the expression of IL-4 mRNA in vitro (Punnonen et al., 1993). In the present study, the production of both cytokines was affected by diet, however, the mitogen was different in both cases.

BBdp animals in general displayed a poor response to mitogens compared to BBn animals. Feeding the FF diet affected the proliferation (estimated by the rate of ^{3}H-thymidine uptake), but not the production of T_h1 cytokines in normal rats. BBdp animals displayed an unexpectedly high mitogen-stimulated production of IFN-γ in splenocytes (Figure 7) and TNF-α in MLN cells (Figure 12), despite the absence of "activation" by the mitogen (demonstrated by a low rate of ^{3}H-thymidine uptake and IL-2 production in splenocytes) . The production of a T_h3 cytokine was not affected by disease or diet, but was produced in all ConA stimulated cells from the spleen, MLN and Peyer's Patches (Figure 13). The above findings suggest that while BBdp animals may not show a normal mitogenic response as indicated by proliferation and production of IL-2 they are still able to produce certain cytokines associated with the pathogenesis of diabetes at levels similar to normal when stimulated with the mitogens ConA or PMA & Iono. The lower production of diabetes "protective" cytokines, IL-4 and IL-10, by BBdp rats may indicate an imbalance between T_h1 and T_h2 cytokine production in stimulated lymphocytes. This is in accordance with the theory that diabetes may result from a biased towards T_h1 cytokine production (Rabinovitch, 1998).

Microbial fermentation of prebiotic FF, such as inulin and fructo-oligosaccharides results in a selective stimulation of lactic acid bacteria (LAB) in the gut (Buddington et al., 1996; Bouhnik et al., 1996; Le Blay et al., 1999; Menne et al., 1999). It is hypothesized that the immunomodulatory properties of FF are a result of increased LAB and the subsequent production of metabolites SCFA's (Schley & Field, 2002). Culturing lymphocytes from humans and mice with LAB has produced contradictory findings with respect to the production of anti-inflammatory cytokines. Some strains of LAB, but not all, stimulate the *in vitro* production of IL-10 by lymphocytes (Sekine et al., 1995; Miettinen et al., 1996; Hessle et al., 1999; Miettinen et al., 1999). One study found that there was an increase in anti-CD3-stimulated IL-10 production in

culture supernatant from peripheral blood mononuclear cells (PMBC) from atopic children fed *Lactobacillus rhamnosus GG* (Pessi *et al*, 2000). This indicates that high colonization of LAB in the gut can influence stimulated peripheral lymphocytes, as suggested by our splenocyte results. It is possible that the increase in the ability to produce IL-10 after ConA stimulation by splenocytes from FF-fed animals is due to short-chain fatty acids, as it has been reported that *in vitro* exposure of PMBC to butyrate significantly upregulated IL-10 production after anti-CD3 stimulation (Saemann *et al*, 2000).

The fermentation of inulin, the FF used in the present study, by whole canine fecal microflora (Vickers *et al*, 2001) and whole human fecal microflora (inoculated in rats fed inulin) (Roland *et al*, 1995), results in significant production of SCFA, in particular butyrate, compared to the feeding cellulose. SCFA resulting from FF fermentation in the colon, have been shown to possess anti-inflammatory properties *in vitro*, (Bohmig *et al*, 1997) (Inan *et al*, 2000;Saemann *et al*, 2000). The anti-inflammatory effects of SCFA's are thought to be attributed to a down-regulation of costimulatory molecules on APC's (Bohmig *et al*, 1997), and/or preventing the action of transcription factors such as nuclear factor of activated T-cells (NF-AT)(Diakos *et al*, 2002). The effects of butyrate on PKC in lymphocytes has not been reported. However, it has been reported that the SCFA butyrate can activate PKC in human K562 erythroleukemia cells *in vitro* (Rivero & Adunyah, 1998). This, however, may not occur in all cell lines (Rickard *et al*, 2000). Evidence suggests that butyrate in combination with PMA may have a synergistic effect on cell differentiation *in vitro* on colonic epithelial cells, although in this particular study, PMA was added to culture prior to butyrate exposure (Rickard *et al*, 1999). The combination of butyrate and PMA has also not been studied with respect to the proliferative response or cytokine production by lymphocytes. It is possible that *in vivo* exposure of lymphocytes to butyrate after animals consumed FF, then subsequent *in vitro* exposure to a PKC-stimulating agent, such as PMA, could result in an enhanced rate of ^{3}H-thymidine incorporation. Future research addressing the effects of butyrate on the intestinal and peripheral immune systems in BBn and BBdp rats should be considered to elucidate the mechanism(s) by which fermentable fibres elicit differential effects on immune function in these animals.

CD80+ Cells in the Gut

We found that distinctive positive staining for CD80 occurred in the cryptic epithelium in all BBn animals. Further investigation revealed that the cells being stained were Paneth cells (Figure 16). The finding that Paneth cells express the costimulatory molecule CD80 has not been reported in the literature, therefore we believe that this is a novel finding. Paneth cells are differentiated epithelial cells that originate from the same intestinal stem cells as absorptive cells, goblet cells and enteroendocrine cells (Porter *et al*, 2002). However, instead of moving and differentiating apically on the villus, they differentiate as they migrate down to the bottom of the crypt (Porter et al., 2001). Each crypt contains approximately 5-15 Paneth cells in total, and numbers do not differ substantially between species (Porter et al., 2002). Diet did not affect the expression of CD80 on Paneth cells from BBn rats.

We found that BBdp animals had consistently no, or little staining in the region where Paneth cells are found (Figure 16). Due to the granularity of Paneth cells, they can be easily distinguished them from other intestinal epithelial cells when stained with eosin (Figure 15) (Porter *et al*, 2002). Examination of hematoxylin and eosin-stained slides revealed that the absolute number of Paneth cells was not significantly different between groups. Therefore, while the absolute numbers of Paneth cells did not differ between BBn and BBdp animals, the absence of CD80 on cells from BBdp rats is a novel finding (Figure 16). The function of CD80 in the gut is not known, but it is of interest that Paneth cells, but not other epitheial cells, express this molecule. Even less clear are the implications of not expressing this molecule as observed in BBdp animals.

We also found unexpected staining of CD80 in the crypts above the region where Paneth cells are located (Figure 17). BBdp rats had a significantly higher proportion of positive staining for CD80 than BBn rats in the cryptic epithelium (Figure 17). This region contains multipotent intestinal stem cells that are in the process of differentiating into the 4 epithelial cell types discussed above (Marshman *et al*, 2002). Among these progenitors are daughter cells that are in separate stages of differentiation (Marshman *et al*, 2002). There are no known cell markers that can definitively identify the stem cells at this time(Marshman *et al*, 2002). The finding that these cells have positive staining for CD80 is novel. Furthermore, the significance of a higher expression of CD80 on intestinal stem cells in the BBdp rat when there appears to be deficient

CD80+ staining on the Paneth cells is of interest. It is possible that BBdp rats possess CD80+ stem cells at early stages of epithelial cell differentiation, but the ability to express CD80 becomes abnormal after full differentiation, as observed in Paneth cells. In addition, the expression of CD80 in the crypts suggests that the immune system might be involved in epithelial cell differentiation.

Dendritic cell (DC) expression of CD80 has been shown to be deficient in BBdp animals, present before the age of disease onset (Delemarre *et al*, 1999). This suggests that there could be a possible overall deficiency in the ability to express this molecule. It is known that CD80 can provide the "second signal" to trigger T-cell activation through ligation of CD28, however, it also plays a role in T-cell tolerance, through ligation of CTLA-4 (Fowler & Powrie, 2002). Thereby, providing a tolerogenic signal to a potential diabetogen. The implications of a CD80 deficiency in the BBdp rat might be a reduced ability to induce tolerance, both to self and dietary antigens. CTLA-4 blockade results in a decrease in tolerance induction (Chen *et al*, 2002). Transgenic NOD mice that are CD80/CD86 deficient develop a more severe incidence of diabetes than control NOD mice, further denoting the importance of costimulatory molecules in self-tolerance (Salomon *et al*, 2000). In contrast, the expression of CD80/86 was reported to be significantly higher on DC from NOD mice than control animals (Marleau & Singh, 2002).

The Development of the Immune System Between 21 and 35 days in Animals Fed Control Diet

Mitogen Stimulated rate of ^{3}H-thymidine Uptake and Cytokine Production

As expected (Field *et al*, 1999a), BBdp rats had lymphopenia present at 21 days of age. As assessed by the rate of ^{3}H-thymidine uptake, cells from BBdp rats responded poorly to ConA. Interestingly, splenocytes from 21-day old BBn rats were able to produce IL-2 at levels similar to 35 day-old rats, despite a reduced rate of ^{3}H-thymidine uptake by ConA-stimulated splenocytes from 21-day old BBn rats. It has been reported that neonatal lymphocytes are able to produce IL-2 at levels similar to adults after mitogen stimulation (Adkins, 1999;Vigano *et al*, 1999). However, proliferation in response to mitogens occurs primarily by mature lymphocytes (Akbar *et al*, 1991). This has been reported in rodents, where splenocytes from neonatal mice had a reduced ability to proliferate in response to ConA and fail to synthesize IL-2 receptors (Lipoldova

et al, 1990). In contrast, the capacity of splenocytes to produce IL-2 after PMA & Ionomycin was higher at 35 days than at 21 days, despite no age effect with this mitogen combination on the rate of ^{3}H-thymidine uptake by splenocytes from both BBdp and BBn rats. This is interesting as it has been reported previously that stimulation of murine neonatal and adult T-cells with a phorbol ester and calcium ionophore resulted in equivalent proliferation and IL-2 production (Adkins & Hamilton, 1992). The altered ability of BBdp lymphocytes to produce regulatory cytokines such as IL-2 at a critical time (weaning) could have implications on gut development, as IL-2 is thought to be important in the development of the gastrointestinal tract during weaning (Thompson *et al*, 1996). The production of IL-10 after ConA stimulation was higher at 35 days than at 21 days by splenocytes from both BBn and BBdp rats. This was expected as studies have reported that there is a decreased ability to produce cytokines by neonatal cells (Sautois *et al*, 1997;Wilson *et al*, 1992).

Although the pattern of IL-2 production was different than the pattern of ^{3}H-thymidine uptake after PMA & Ionomycin stimulation (Figure 20), the production of IFN-γ after stimulation with PMA + Iono was similar in BBn and BBdp splenocytes (Table 15). BBdp splenocytes demonstrated a reduced ability to respond to this mitogen, when the extent of activation is defined by the rate of ^{3}H-thymidine uptake and IL-2 production. The ability to produce high levels of IFN-γ in the absence of a vigorous mitogenic response suggests that BBdp immune cells can produce this cytokine, which is involved in the pathogenesis of diabetes, with less stimulation, even before weaning. IFN-γ production by MLN cells from 21-day old BBn rats was higher than at 35 days (Table 17). In contrast, Con-A stimulated ^{3}H-thymidine uptake was higher at 35 days than at 21 days in BBn rats (Table 16). Interestingly,unlike our BBn findings, it was reported that human neonatal cells, have a lower ability to produce IFN-γ compared to adult cells (Sautois *et al*, 1997). However, this lower production of IFN-γ, after stimulation, by immune cells from human neonates, may depend on the diet of the infants (Field et al, unpublished results). Despite the increase in the production of IFN-γ at 35 days, MLN cells from BBdp rats still demonstrated a lower rate of IFN-γ production in response to ConA compared to BBn rats. In contrast, ConA-stimulated TNF-α production by MLN cells did not differ amongst groups. This pattern of cytokines in BBdp rats is suggestive of an increased T_h1 type response. A $T_h1$cytokine pattern is thought to be involved in diabetes pathogenesis (Rabinovitch, 1998). This pattern of response is similar at 21 and 35 days of age, suggesting that a defect in cytokine secretion after lymphocyte activation is present early in life

In conclusion, the reduced ability to respond to mitogens (as indicated by the lower incorporation of ^{3}H-thymidine, IL-2 production, and IFN-γ production by MLN cells) by BBdp rats supports a functional abnormality in immune function in these animals that is present early in life, prior to the development of diabetes.

Immunohistochemistry (IgA+ and CD80+ cells)

Consistent with previous findings from our lab (Butler S, 1999), IgA+ cells in the LP were not detected in either BBn or BBdp animals at 21 days-of-age. At 35 days-of-age, IgA+ cells were visible in the lamina propria in numbers not different between strains fed the control diet. Thus, it can be concluded that IgA+ cells in the lamina propria begin to appear sometime between 21 and 30 days. It has been reported in the literature that IgA+ cells were detected in between 23-25 days after birth (Umesaki & Setoyama, 2000). Our results support the hypothesis that the development of of IgA+ cells in the LP of the small intestine is diet (antigen)-dependent (Crabbe et al., 1968).

As hypothesized, BBn and BBdp animals displayed a differential immune development, as measured by CD80+ expression on Paneth cells at 21 and 35 days. Although there was no difference in the total number of Paneth cells in each crypt, there was a 6–fold increase in CD80+ Paneth cells in BBn rats between 21 and 35 days of age (Figure 25). Positive staining in the lower third of the crypt above the region where Paneth cells are found was different between BBdp and BBn rats fed control diet. The proportion of CD80+ staining in this region was significantly lower in BBn rats at 35 days than at 21 days, suggesting that this is a change that occurs with maturation of the gut. BBdp rats had similar proportions of stained area at both ages. As discussed earlier, this area represents the region in which intestinal stem cells are found (Marshman *et al*, 2002). The failure of BBdp rats to express CD80 on Paneth cells, and the overexpression (or the failure to down-regulate expression) of CD80 in the stem cell region during the 2 weeks post-weaning further supports a developmental immaturity in these animals. These findings should be followed up in subsequent experiments.

Conclusion

It is not known whether the presence of FF in the diet potentiates diabetes in the BB rat. However, the immunomodulatory properties of consuming FF, as found in the current study, suggest that it may somehow play a significant role in altering the development of the gut and immune system in the BB rat. Furthermore, maladaptive processes to dietary challenge in the BB

rat appear to be exacerbated by fermentable fibre. These changes may increase the potential antigen exposure in the gut. In a normal, healthy animal, the gut and GALT communicate via intraluminal signals such as cytokines and growth hormones and the resulting IgA synthesis to limit dietary antigen exposure to the peripheral immune system. The high number of IgA+ cells in BBdp animals provides support for an increased immune exposure, a compromised barrier function, or inability to produce or receive intraluminal signals in response to dietary change.

The lack of association between cow's milk and diabetes in human studies points to a new direction in research to link diabetes incidence within the human population. Early weaning from breast-milk to solid-foods, primarily wheat and gluten-containing grains, could be a more important contributor to diabetes incidence in human populations than exposure to cow's-milk protein. Recent studies have implicated wheat-based chow diets and gluten in increased incidence of diabetes in BB rats and NOD mice (Beales *et al*, 2002) and increased autoantibodies to wheat proteins are reported in human subjects with diabetes compared to non-diabetic controls (MacFarlane *et al*, 2003). Wheat-based cereals contain fermentable fibre, and if an inability to adapt to fermentable fibre could be extended to humans, our data could support the early weaning-food theories suggested from epidemiological data. The fibre fed in this study was inulin, originating from jerusalem artichokes. However, β-glucan, a fermentable and potentially prebiotic fibre found in all gluten-containing grains has also been shown to have immunomodulatory effects (Yun *et al*, 1997;Yun *et al*, 1998;Yun *et al*, 2003). The implication that dietary fermentable fibres have the abililty to modulate immune function in autoimmune disease may have future implications on infant-feeding practice recommendations, particularly in those with a genetic susceptibility to diabetes.

Future Research

Future research elucidating the mechanisms for diet adaptation in the BB rat may offer insight into preventative treatment for autoimmune disease in which an oral antigen may be involved. From the present study, several questions have been raised. Mechanisms by which fermentable fibre affect immune cells in BBn and BBdp animals are not known. We have demonstrated that feeding FF impacts the peripheral and gut immune systems, but the connection between the two systems was not explored. To answer this question, feeding studies with LAB added to a semi-purified diet with or without FF might help us to understand if LAB do indeed increase antigen exposure to the immune system in both BBn and BBdp animals. Infusion of

SCFA into the portal vein of BBdp and BBn animals and subsequent *in vitro* measures of immune function could indicate the immune response to systemic exposure of SCFA. Infusion into the small intestine or large intestine might give more of an indication of GALT response to SCFA. Furthermore, epithelial cell regulation of portal entry of SCFA would then be taken into consideration. *In vitro* exposure of BBdp and BBn lymphocytes might help determine the functional response to butyrate, as reported in the literature (Bohmig *et al*, 1997;Saemann *et al*, 2000).

No diet effects were observed in unstimulated lymphocyte subsets from the spleen or MLN. It would be of interest to look at the phenotype distribution of stimulated cells. By measuring the proportion of different lymphocyte subsets pre- and post activation, it might be possible to determine which cell subsets are not responding normally to mitogen stimulation in BBdp animals fed FF. It would also be of interest to measure the expression of activation markers after mitogen stimulation by flow cytometry in BB rats fed FF. Measuring the expression of gut homing molecules, normally expressed on mature lymphocytes (Schweighoffer *et al*, 1993), might provide some information about lymphocyte migration patterns, especially if differences are observed in the periphery between diets and strains.

Another question raised during this study was whether the gut immune system in BBdp rats fed FF is at increased exposure to luminal contents due to greater intestinal permeability, either through increased M-cell and dendritic cell endocytic activity, or by a compromised epithelial barrier. Assessing intestinal permeability, *in vivo*, and *ex vivo* might offer some insight as to the size of macromolecules that are able to pass through the intestinal epithelial layer. In addition, markers of inflammation, such as IFN-γ mRNA could provide further evidence of intestinal permeability (Sutas *et al*, 1997).

Addressing the higher IL-10 secretion in both BBn and BBdp rats after FF feeding could be of interest in other inflammatory disorders, such as inflammatory bowel disease (IBD). Oral administration of LAB has been shown to improve measures of inflammation in experimental models of IBD (Madsen *et al*., 1999);(Cherbut *et al*, 2003). Feeding FF (the pre-biotic) could be another strategy to probiotics of increasing LAB in the gut.

Finally, the finding that Paneth cells in the gut differentially express CD80 between BBn and BBdp rats deserves more attention. To study the differences in Paneth cell function in the BB rat, it would be necessary to examine primary tissue, isolating intestinal crypts, as Paneth cell-

derived cell lines are not available (Porter *et al*, 2002). As CD80 and MHC class II are often co-expressed on antigen-presenting cells, it would be of interest to compare the expression of MHC class II in the gut between BBdp and BBn animals. It would be of interest to discern if LAB can modulate Paneth cell function, and therefore increase or decrease the amount of antimicrobial acitivity in the lumen. Also interesting would be the ability of Paneth cells *in vitro* to stimulate lymphocyte activity in BBn and BBdp animals, to characterize their functions as antigen presenting cells. Normal intestinal epithelium functions to induce tolerance to potential dietary and other antigens. However, T-cells at the base of the crypt may be able to be activated by Paneth cells, as CD80 expression in the presence of MHC class II is able to create the second signal necessary for T-cell tolerance. In addition, it would be of interest to look at co-stimulatory molecules on T-cells. The expression of CTLA-4 and CD28 would give an indication of T-cell function in the gut, as CTLA-4 is involved in tolerance and CD28 is involved in activation. Additionally, evidence exists for mucosal inflammation in the small intestine of BBdp rats prior to the age of diabetes onset (Hardin et al., 2002). In patients with IBD and *helicobactor pylori* infection, it was found that an increased expression of CD86, but not CD80, in the intestinal mucosa was associated with inflammation (Ye et al., 1997; Nakazawa et al., 1999) Therefore, measuring CD86 in the gut could give a better indication of inflammation than CD80 in our model.

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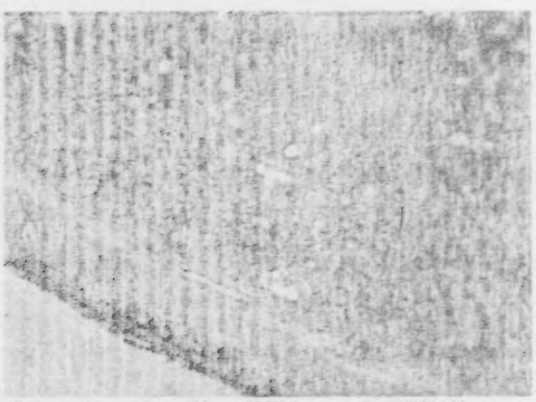
Appendix A: Samples of Immunohistochemical Staining

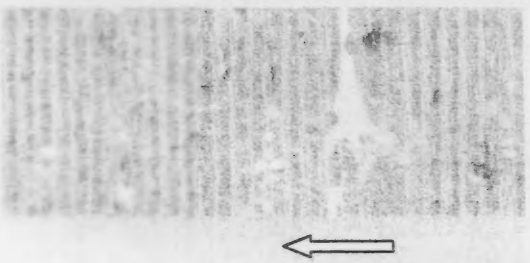
Appendix Figure 1 Jejunal Section of BBn rat fed control diet

(Control slide: primary CD80Ab+ blocking peptide) No positive staining

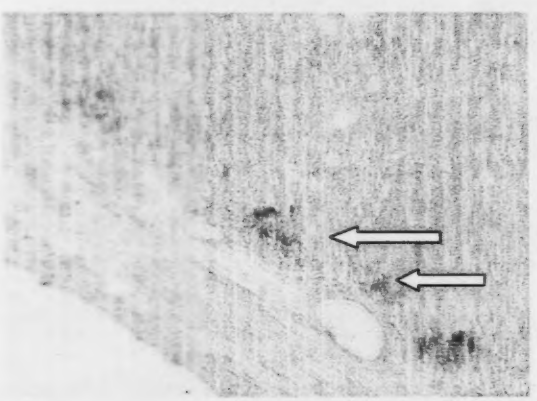


Appendix Figure 2 Jejunal section from BBdp rat fed FF diet

(Control slide: primary CD80 Ab + blocking peptide) No positive staining

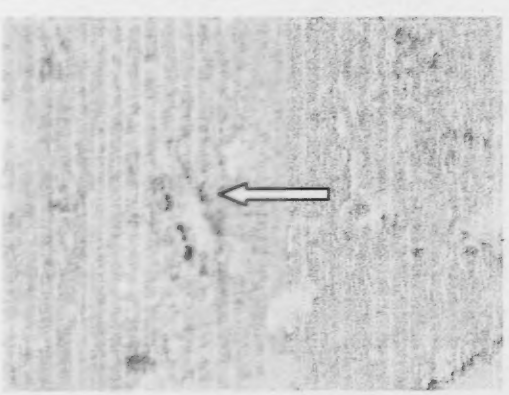


Appendix Figure 3 Jejunal Section from BBn rat 35 days old fed FF-CD80+ staining in Paneth cell Region



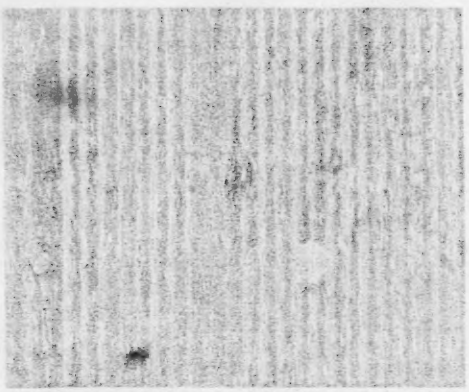
Appendix Figure 4 Jejunal Section from BBn rat 35 days old fed control

CD80+ staining in Paneth cell region

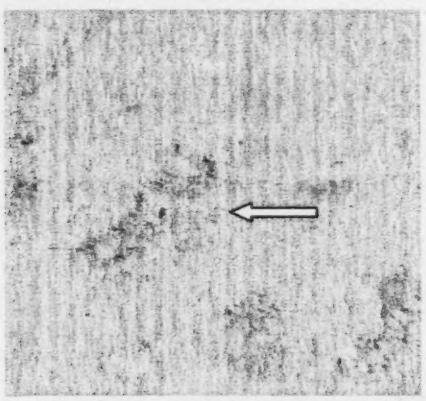


Appendix Figure 5 Jejunal section from BBdp rat 35 days old fed FF

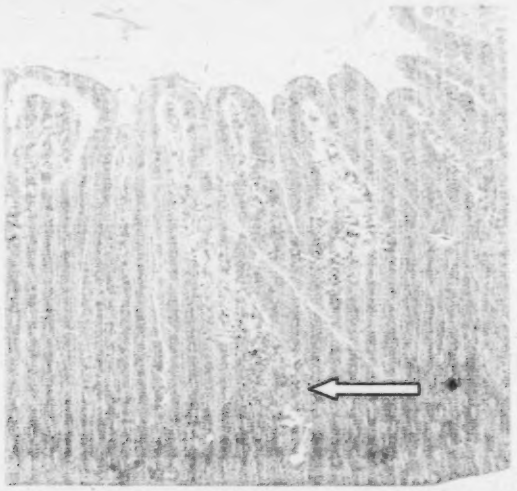
CD80+ staining in stem cell region



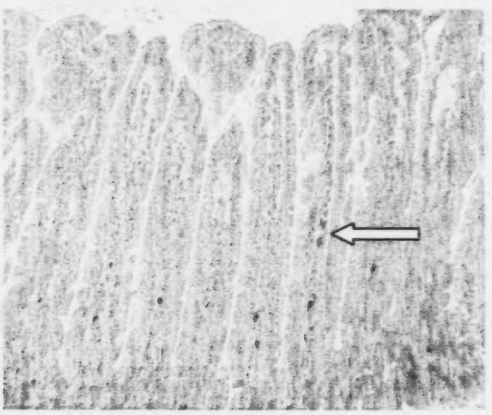
Appendix Figure 6 Jejunal Section from BBdp rat 35 days old fed Control diet
CD80+ staining in stem cell region



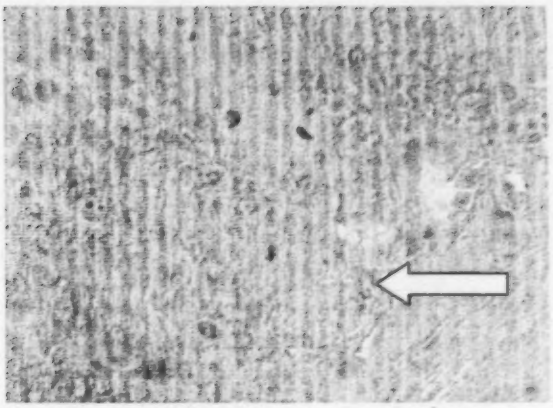
Appendix Figure 7 Jejunal Section from BBdp rat 21 days old



Appendix Figure 8 IgA staining in the lamina propria from a BBn rat Fed FF



Appendix Figure 9IgA Staining in the lamina propria from a BBdp rat fed FF diet

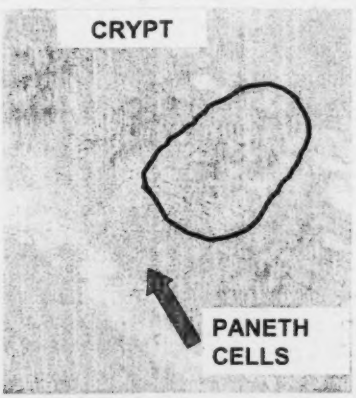


Appendix Figure 10 Hematoxylin and Eosin Stained Slides showing Paneth cells at base of crypt

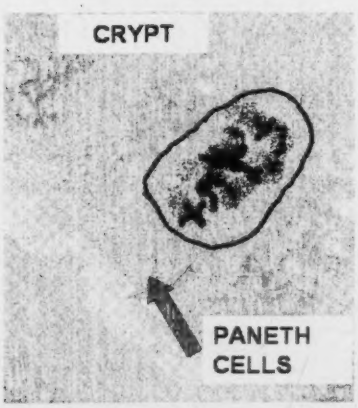
Appendix B Metamorph Procedure for Measuring Proportion of Positive CD80 staining in the Lower Third of the Crypt in the Region Above the Paneth Cells.

Step 1: Take picture of slide (with at least 1 full crypt) at 500x.
Step 2: Go to Display, click on Colour Separate, Click "add to" beside green image
Step 3: Switch green image to monochrome by clicking on the colour icon on the right
Step 4: Outline lower third of crypt above the Paneth cells (see figure 11)
Step 5: Go to threshold toolbar on the right side of image and click on inclusive threshold
Step 6: Set threshold level at approximately 1/3 from the bottom (analyse slides that were stained together, since colour may vary slightly from day to day) until all darkest areas are filled with red (see figure 12-black fill).
Step 7: Go to Measures and click on region statistics
Step 8: Record total area, thresholded area and % thresholded area.

Note: Analysis was done using 9 crypts/sample.



Appendix Figure 11: Outlining crypt



Appendix Figure 12: Thresholded crypt.